IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Applicants	:	David K. Gong, et al.	
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TC/A.U.	:	1616	
Examiner	:	Alstrum-Acevedo, J.H.	
Docket No.	:	31176282-004001	
Customer No.	:	51738	
Entitled	:	Hemophilia Treatment by Inhalation of Coagulation Factors.	

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Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

AMENDED APPEAL BRIEF PURSUANT TO 37 C.F.R. §1.191

Appellants filed the Appeal Brief on January 28, 2008. In response to the Notification of Non-Compliant Appeal Brief mailed February 20, 2008, Appellants file this amended Appeal Brief. Appellants take this appeal from the Final Office Action mailed October 26, 2007, and the Advisory Action, mailed January 11, 2008. The deadline for the response to the Notification of Non-Compliant Appeal Brief is March 20, 2008. Therefore, this Amended Appeal Brief is timely filed.

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1. **REAL PARTY IN INTEREST**

The real parties in interest are Nektar Therapeutics and Wyeth.

2. RELATED APPEALS AND INTERFERENCES

Appellants know of no other appeal or interference that will directly affect, or be directly affected by, or that will have a bearing on the Board's decision in the pending appeal.

3. STATUS OF CLAIMS

Claims 29, 32-33, 36-37 and 40 are currently pending. The remaining claims (1-28, 30-31, 34-35, 38-39 and 41-52) have been cancelled.

Claims 29, 32-33, 36-37 and 40 were rejected in the Final Office Action, October 26, 2007, and the rejection of claims 29, 32-33, 36-37 and 40 is herewith appealed. Amendments were submitted in a Response to Office Action, December 21, 2007. The amendments were entered, pursuant to the Advisory Action, January 11, 2008, but all claims remained rejected under 35 U.S.C. § 103 over Lechuga (attached at Appendix B, Exhibit 3). Therefore, no amendments are pending, and a clean copy of the appealed claims are submitted in APPENDIX A.

4. STATUS OF AMENDMENTS

Amendments were submitted in a Response to Office Action, December 21, 2007. The amendments were entered, pursuant to the Advisory Action, January 11, 2008, and no further amendments are outstanding.

5. SUMMARY OF CLAIMED SUBJECT MATTER

The invention is directed to methods of treating hemophilia (Abstract).

Prior art methods for treating hemophilia requires frequent injections of the clotting Factor IX, herein known as FIX. This treatment, however, has serious drawbacks. First, injected or intravenous medications are unpleasant for the patient, requiring the use of needles. Additionally, the method is inconvenient because one typically must go to a clinic in order to obtain treatment. More seriously, the pharmacokinetics of intravenous FIX (see Specification, Figure 8, attached at Appendix B, Exhibit 1) are sub-optimal, resulting in a large initial dose of FIX, which can result in serious complications and even death due to inappropriate clotting. Then, the FIX is cleared very quickly from the bloodstream (Specification, Figure 8), necessitating frequent administration or restriction to administration on demand (e.g., when bleeding occurs).

The inventors have improved FIX treatment in many respects with the development of an inhaled form of FIX (see Abstract). The inhaled FIX avoids the use of needles and is much more convenient for the patient (Specification ¶ 10). Prior to the invention herein, no-one had ever successfully administered FIX by inhalation, and its successful delivery was a surprise, since the prior art inhaled proteins were all of significantly smaller size. (Specification ¶ 18-24). This is the first demonstration of inhaled delivery of FIX, and the first time a protein this large has been successfully delivered via the lung (Specification ¶ 19-20).

The only other person that tried to develop an inhaled FIX was unsuccessful because 50% of the FIX was denatured during the process (see Gupta, Appendix B, Exhibit 2, also Specification ¶ 19-20). Lechuga developed a dry powder FIX, but failed to test the FIX powder for enzymatic activity nor use it in any treatment method (See Lechuga at Appendix B, Exhibit 3).

Inhaled FIX has surprisingly improved pharmacokinetics over the intravenous form. Inhaled FIX surprisingly **avoids** the large initial bolus of FIX, **and** provides steady state FIX levels for **more than 100 hours** (Figure 8). Therefore, the inhaled FIX of the invention can be used prophylactically, e.g., to prevent bleeding episodes with a convenient inhaled form of the

drug. (See Specification ¶ 22.) Not only does the inhaled FIX last longer, but it is safer for regular use since the large bolus of clotting factor is avoided (Figure 8).

More particularly, the invention relates to a method of preventing hemophilic bleeding in advance of a bleeding event by aerosolizing a monomeric FIX, wherein the aerosolized monomeric FIX has a mass median aerodynamic diameter of between 2 and 4 μm, a fine particle fraction less than 3.3 μm of at least 50%, is at least 90% monomeric, wherein the after-aerosolization activity/pre-aerosolization activity is at least 80%, and is a dry powder having less than 10% water (wt/wt) (and lacks ethanol). The aerosolized monomeric and active FIX is slowly maximally inhaled by the patient, allowing the FIX to deposit in the deep lung tissue such that it is sequestered to provide sufficient FIX to prevent bleeding for at least 100 hours after administration. This methodology allows dosing on a weekly basis. (See claims 29, 33 and 37 and 40; the elements of each independent claim are discussed in the Specification at ¶ 12, lines 1-3; ¶ 21 lines 1-7; ¶ 22, lines 1-3; ¶ 23, lines 1-6; ¶ 30, lines 1-6; ¶ 40, lines 1-5; ¶ 63, lines 1-2; ¶ 85, lines 1-3; ¶ 90, lines 2-3; and Figure 8.)

Additionally, the invention relates to treatment methods using aerosolized monomeric active FIX that was prepared by diafiltering concentrated FIX solution to approximately 12 mg/ml, spray drying the diafiltered solution between 40-60 psi, and 60-70°C at 5 ml/min and approximately 17.8 standard cubic feet per minute. The spray dried active monomeric FIX is then transferred to a sealed storage container at less than 5% relative humidity. (See claims 32 and 36, and Example 2, ¶ 59-83; the elements of each dependent claim are discussed in the Specification at ¶ 22, lines 1-6; ¶ 61, lines 3-7, ¶ 63, lines 1-2; and ¶ 64, lines 1-2.).

Even more particularly, the invention relates to pending claims 29, 32-33, 36-37 and 40 (See Appendix A).

6. **GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

Claims 29, 33, 37 and 40 are rejected as obvious under 35 U.S.C. § 103 over Lechuga (attached at Appendix B, Exhibit 3), which teaches a dried FIX powder having the recited MMAD, and in view of Kurachi (attached at Appendix B, Exhibit 5), which teaches that the natural *in vivo* form of FIX is monomeric.

All other recited claim elements are argued to be **inherent** to Lechuga.

Claims 32 and 36 are rejected as obvious under 35 U.S.C. § 103 over Lechuga in view of Kurachi in further view of Huang (attached at Appendix B, Exhibit 4) which allegedly teaches diafiltration of FIX.

No other rejections are outstanding.

7. ARGUMENTS TO REBUT REJECTION BASED ON 35 U.S.C. § 103

Claims 29, 32-33, 36-37 and 40 are currently pending. Claims 29, 33, 37 40 are rejected as obvious under 35 U.S.C. § 103 over Lechuga in view of Kurachi. Claims 32 and 36 are obvious under 35 U.S.C. § 103 over Lechuga in view of Kurachi in further view of Huang.

Appellants appeal the rejection, as follows:

A. *PRIMA FACIE* OBVIOUSNESS CASE

The *prima facie* obviousness burden lies on the Examiner to show at least the following: 1) that the art teaches every element of the claimed invention,[1] 2) that there is a motivation to combine or modify the art,[2] and 3) that there is a reasonable expectation of success in making that combination or modification. While the expectation of success need not be absolute, there does need to be a **reasonable** expectation of success. *Takeda Chem. Indus. v. Mylan Labs., Inc.*, 417 F. Supp. 2d 341, 371 (Fed. Cir.2006) ("While a reasonable expectation of success must be shown, in order to show *prima facie* obviousness it is not necessary to show that success was absolutely predictable."); *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991) (discussing the obviousness case and stating that one element is "whether the prior art would also have revealed that . . . those of ordinary skill would have a reasonable expectation of success.").[3]

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[1] *See e.g.*, MPEP 2143.03 ("All Claim Limitations Must Be Taught or Suggested").

[2] KSR did not negate the motivation to combine test, but only cautioned against its rigid application. *KSR Int'l Co. v. Teleflex Inc.*,127 S. Ct. 1727, 1741 (U.S. 2007) ("When it first established the requirement of demonstrating a teaching, suggestion, or motivation to combine known elements in order to show that the combination is obvious, the Court of Customs and Patent Appeals captured a helpful insight. . . . a patent composed of several elements is not proved obvious merely by demonstrating that each of its elements was, independently, known in the prior art.. . . it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does.").

[3] *See* also *MPEP* 2143.02 entitled "Reasonable Expectation of Success Is Required".

If the *prima facie* case is made, it can be rebutted by showing long felt need, commercial success, unexpected results,[4] or teaching away. *See e.g., KSR Int'l Co. v. Teleflex Inc.*,127 S. Ct. 1727, 1734, 1740 (2007).

B. SEQUESTRATION AND DOSAGE ELEMENTS MISSING

Examiner has failed to cite **any** art describing the missing elements "allowing said monomeric FIX to deposit in the deep lung tissue such that said monomeric FIX is sequestered in said deep lung tissue" and "providing sufficient FIX to prevent bleeding for at least 100 hours after administration." **Examiner cannot cite to art teaching sequestration or 100 hour dosing because these elements did not exist prior to Applicants invention.** Prior to Applicants invention the dosing regime was much more frequent due to the rapid clearance of FIX from the blood, and sequestration of FIX did not exist.

Even in the wake of *KSR Int'l Co. v. Teleflex Inc.* (127 S. Ct. 1727 (U.S. 2007)), Applicants know of no legal principle that suggests that a *prima facie* case can be maintained where two claimed elements are not found in the prior art. Thus, in the absence of these elements, the obviousness rejection cannot be maintained because the *prima facie* case is not made.

C. SEVERAL MISSING ELEMENTS

In addition to the sequestration and dosage elements, the following elements are also not found in the cited art:

i) preventing hemophilic bleeding in advance of a bleeding event
ii) at least 90% monomeric after-aerosolization
iii) 80% activity retained after-aerosolization

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[4] *Takeda Chem. Indus. v. Mylan Labs., Inc.*, 417 F. Supp. 2d 341, 371 (Fed. Cir.2006) ("While a reasonable expectation of success must be shown, in order to show *prima facie* obviousness it is not necessary to show that success was absolutely predictable."); *In re Vaeck*, 947 F.2d 488, 493 (Fed. Cir. 1991) (discussing the obviousness case and stating that one element is "whether the prior art would also have revealed that . . . those of ordinary skill would have a reasonable expectation of success."). *See also*, MPEP 716.02 (a) ("Evidence of unobvious or unexpected advantageous properties, such as superiority in a property the claimed compound shares with the prior art, can rebut *prima facie* obviousness.").

iv) does not have ethanol
v) slowly maximally inhaling[5]

All of these elements are merely **assumed** to exist in Lechuga or are allegedly obvious based on Lechuga, even though the formulation taught therein is **not identical** and even though Lechuga goes no further than making a dried powder, and **neither** tests its activity, **nor** uses it in any treatment.

D. KURACHI IRRELEVANT

It is possible that Examiner no longer maintains the rejection based in part on Kurachi, since Kurachi is not mentioned in the Advisory Action. To the extent that this Examiner continues to rely on Kurachi, it is distinguished as discussing *in vivo* FIX. *In vivo* structure tells us nothing about what the dried, aerosolized FIX will look like and it is a **well known fact** that many proteins **clump or otherwise deteriorate** on such treatment.[6] Therefore, Examiner's **assertion that dried, aerosolized FIX is a monomer based on the teachings of Kurachi is illogical.**

Further, since Kurachi discusses *in vivo* activity, there is no reason to combine it with the teaching of Lechuga, which is directed to powdered proteins.

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[5] Examiner argues that because Applicants have shown that "slowly maximally inhaling" is a well understood term, that no references need be provided to show this element. This conflates definiteness with obviousness and under this standard anything written in "plain English" would be obvious. *See* Advisory Action, 1/11/2008, p. 2.

[6] *See e.g.*, Gupta, *et al.*, Pulmonary Delivery of Human Protein C and Factor IX Oxygen Transport to Tissue XVIII, Chapter 55, p. 429-435 (1997) ("Formulation of proteins into MDI's [metered dose inhalers] and DPI's [dry powder inhalers] is a **formidable** task because dehydration and subsequent communition of proteins to produce powders in the size range suitable for inhalation may lead to **loss of activity**.") (emphasis added); Choi et al., Inhalation delivery of proteins from ethanol suspensions, Applied Biological Sciences 98(20): 11103-11107 (2001) ("protein powders, at least as conventionally made, are liable to **clump** formation"); Huang, *et al.*, US6280729 ("most purification procedures result in considerable proteolytic activation and/or degradation of factor IX") (Exhibits 2 and 4). Note: Neither activation nor degradation are desirable—activated protein causes undesired clotting complications.

E. HUANG TEACHES AWAY

Examiner cites Huang for teaching the routine nature of FIX diafiltration. Huang is actually directed to stabilizing FIX using salts ("The present invention is based upon the recognition that water-soluble organic and inorganic salts can be used to protect factor IX from proteolysis . . ."). In fact, Huang compares his method directly against a method including diafiltration, and concludes that his high salt method is much improved (see Table 1 and discussion at Example 2). In fact, Huang shows that diafiltration resulted in less than 10% FIX.[7] Thus, Huang fairly be said to teach away from diafiltration. Therefore, citation of Huang against pending claims 32 and 36 is not appropriate, and these claims (at least) are not obvious.

Huang also teaches the difficulty in preparing pure active FIX. Huang states that "most purification procedures result in considerable proteolytic activation and/or degradation of factor IX." (col. 5, lines 14-16). Thus, Huang actually establishes that the part of preparing active FIX is unpredictable and prone to failure, thus again demonstrating the inappropriateness of Examiner's assumptions about the qualities of the FIX in Lechuga.

Neither Huang, nor the other cited art, teach the specific recited conditions of diafiltering concentrated FIX solution to a concentration of approximately 12 mg/ml, spray drying at 40-60 psi and 60-70°C at 5 ml/min and 17.8 standard cubic feet per minute and transferring spray dried FIX to a sealed storage container at less than 5% relative humidity. Lechuga does not provide spray drying conditions, except to say that spray drying can occur between 30-200°C (page 17, line 31 to page 18, line 3), nor does it provide any details about FIX preparation and aerosolization, except to say that it is spray dried and provided ingredients (Example 7 at page 44). Therefore, at the very least, claims specifically recited preparation conditions not taught in the prior art (claims 32 and 36) should be allowable.

Finally, since Huang discusses preparation of liquid FIX, there is no reason to combine it with the teaching of Lechuga, which is directed to powdered proteins.

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[7] *See also*, Huang, col. 4, lines 47-50 (method of US4447416 including diafiltration "leads to a factor IX product in which **less than 10% of the protein is factor IX**, more than 90% of the material consisting of contaminating protein species.").

F. FORMULATIONS NOT IDENTICAL, THUS RECITED ELEMENTS STILL MISSING

Examiner asserts that the observation of unknown properties of the Lechuga formulation are not novel. However, the preparation method of Lechuga is not provided and the formulations are **not** identical:

Lechuga	Closest Formulation Described in 10/820,656
37% FIX, 3% NaCitrate, 60% Leucine	32.6% FIX, 7.4 % NaCitrate, 60% Leucine
56% FIX, 4% NaCitrate, 40% Trileucine	52.6% FIX, 7.4 % NaCitrate, 40% Trileucine

One cannot **assume** that the Lechuga FIX has the requisite properties **because the formulations were not identical** and the preparation method unknown. There is simply no way to get from Lechuga to an aerosolized FIX having the recited characteristics and providing sequestration and at least 100 hrs of dosing per the recited treatment claims without several assumptions and these assumptions cannot be properly made under established inherency principles (as shown below). Nor is it proper to assume the properties of FIX when the art teaches the difficulty in preparing FIX in a variety of ways.

G. INHERENCY USED TO PROVIDE SEVEN MISSING ELEMENTS

Inherency can be used to supply a missing element that is not expressly taught in the art, but which nonetheless **must** be present and would be so understood to be present in the prior art. *Astra Aktiebolag v. Andrx Pharms., Inc.*, 483 F.3d 1364, 1373 (Fed. Cir. 2007) ("a prior art reference without express reference to a claim limitation may nonetheless anticipate by inherency."); *EMI Group N. Am., Inc. v. Cypress Semiconductor Corp.*, 268 F.3d 1342, 1350 (Fed. Cir. 2001) ("A prior art reference anticipates a patent claim if the reference discloses, either expressly or inherently, all of the limitations of the claim.") (cite omitted).

However, Applicants know of **no** case where inherency was used to provide at least seven of eleven claimed elements:

#	Claimed Invention Per Claim[8]	Lechuga
1.	Preventing hemophilic bleeding in advance of a bleeding event	General treatment use suggested ("useful in the treatment of hemophilia B"), BUT "advance" or "prophylactic" treatment **not** taught or exemplified
2.	Aerosolizing monomeric Factor IX (FIX)	See Table 15 showing powdered FIX (does **not** show monomer, see 90% monomer element below)
3.	MMAD of between 2 and 4 μm	See Table 15 showing that powdered FIX has 2-4 MMAD
4.	FPF % < than 3.3 μm of at least 50%	Lechuga teaches FPF generally, but **not** in relation to FIX and **NOT** the same as that claimed. "The powders of the invention . . . possess FPF values ranging from about 35%-85%. Such powders contain at least about 35 percent of aerosol particle sizes below 3."
5.	90% monomeric	Aerosolized monomers neither taught nor mentioned in Lechuga
6.	After-aerosolization activity is at least 80%	Post-aerosol activity neither taught nor mentioned in Lechuga
7.	Less than 10% water (wt/wt)	Water content of FIX neither taught nor mentioned in Lechuga, but Lechuga states generally that the moisture content of a dry powder is low ("'Dry powder' refers to a powder composition that typically contains less than about 20% moisture...most preferably contains less than about 3% moisture, depending upon the particular formulation."). Therefore, unknown FIX water content **may be higher** than that claimed. Lechuga also teaches the use of liquid formulations that are preferred ("The compositions described herein may be in powdered form or may be flowable liquids. **Liquid formulations are preferably solutions in which the active drug is dissolved in a solvent** (e. g., water, ethanol, ethanol-water, saline)").
8.	Does not have ethanol	Ethanol content of FIX not taught. Instead **teaches away** ("Liquid formulations are preferably solutions in which the active drug is dissolved in a solvent (e. g., water, **ethanol**, ethanol-water, saline)", also "The aqueous formulation may optionally contain additional water-miscible solvents, such as acetone, **alcohols** and the like."). However, Applicants have shown that this degrades FIX, and thus avoidance of ethanol is a claimed element. Lechuga does not provide the actual solvents used to solubilize FIX, and Examiner assumes they are water based.
9.	Slowly maximally inhaling aerosolized monomeric FIX	Slow maximal inhalation is not neither taught nor mentioned in Lechuga. Inhaled delivery of drugs in general is taught, but slow maximal inhaled delivery of aerosolized monomeric FIX to lungs is not taught.
10	Allowing said monomeric FIX to deposit in the deep lung tissue such that said monomeric FIX is sequestered in said deep lung tissue	Single statement regarding delivery to the "deep lung" is present, but not in relation to FIX, nor is sequestration in the lung taught or mentioned in Lechuga.
11	To provide sufficient FIX to prevent bleeding for at least 100 hours after administration.	100 hour dosage neither taught nor mentioned in Lechuga. **Prior art teaches frequent use necessary**. See BeneFix package insert.

[8] Only claim 1 is used herein for brevity, but the exercise could easily be repeated with every other claim and Applicant does not imply thereby that the remaining claims stand with claim 1. Each must be examined on its own merit.

Thus, Examiner assumes **seven of eleven** elements are present based on the inherent teachings of Lechuga. This seems to be stretching inherency principles to near invisibility.

H. ALLEGEDLY INHERENT ELEMENTS NOT "NECESSARILY PRESENT"

Inherency cannot be based on what **might or might not** be present. *In re Oelrich,* 666 F.2d 578, 581-82 (C.C.P.A. 1981) ("To establish inherency, the extrinsic evidence 'must make clear that the missing descriptive matter is **necessarily present** in the thing described in the reference . . . Inherency, however, may not be established by probabilities or possibilities . . .") (emphasis added). Here, since the formulations are **not** identical and the method of preparation **not** described, one cannot **assume** that the requisite properties were in fact present. There is simply no basis to assume that the powder of Lechuga has all of the recited characteristics and would be sequestered in the lung sufficient to provide for at least 100 hours of dosing.

I. ASSUMPTIONS NOT REASONABLE

Examiner asserts that the formulations of Lechuga are "substantially the same" and that "Lechuga's FIX dry powders are **deemed** to have the same properties."[9] Even though the prior art shows that drying powders causes degradation[10] and that the **only** prior attempt to aerosolize liquid FIX resulted in 50% denaturation,[11] the Examiner argues that he "reasonably expected that the prior art compositions upon administration by inhalation would exhibit the same depot effect." Until the Examiner declares under oath that such alleged facts are true, these statements are mere argument, and can be (and have been) rebutted by competent evidence (prior art

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[9] Office Action, 10/26,2007, p. 6 (emphasis added).

[10] *See e.g.*, Gupta, *et al.*, Pulmonary Delivery of Human Protein C and Factor IX Oxygen Transport to Tissue XVIII, Chapter 55, p. 429-435 (1997) ("Formulation of proteins into MDI's [metered dose inhalers] and DPI's [dry powder inhalers] is a **formidable** task because **dehydration and subsequent communition of proteins** to produce powders in the size range suitable for inhalation may lead to **loss of activity**.") (emphasis added); Huang, *et al.*, US6280729 ("most purification procedures result in considerable proteolytic activation and/or **degradation** of factor IX") (Exhibits 2 and 4) (emphasis added).

[11] Gupta, *et al.*, Pulmonary Delivery of Human Protein C and Factor IX Oxygen Transport to Tissue XVIII, Chapter 55, p. 429-435 (1997) ("in the process of being aerosolized human Factor IX is **50% denatured** at the air water interface.") (emphasis added).

publications) proving that the art is not as predictable as assumed by the Examiner. *See e.g., Brand v. Miller,* 487 F.3d 862, 869 (Fed. Cir. 2007), ("We therefore hold that, in the context of a contested case, it is **impermissible** for the Board to base its factual findings on its [own] expertise, rather than on **evidence in the record**, although the Board's expertise appropriately plays a role in interpreting record evidence.") (emphasis added).

J. NO REASONABLE EXPECTATION OF SUCCESS

Examiner merely assumes that the FIX of Lechuga is monomeric and fully active. Lechuga, however, does not test the protein for form or activity. Further, the prior art actually establishes that it's **not** reasonable to assume success. Gupta, for example, states "dehydration and subsequent communition of proteins to produce powders in the size range suitable for inhalation may lead to loss of activity" and his own attempt to aerosolize FIX destroyed half of it.[12] Huang too states that "most purification procedures result in considerable proteolytic activation and/or degradation of factor IX."[13] Other publications also teach that drying proteins causes clumping.[14] These published statements establish that there is no reasonable expectation of success.

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[12] *Id.*, also ("dehydration and subsequent communition of proteins to produce powders in the size range suitable for inhalation may lead to loss of activity.").

[13] Huang, *et al.*, US6280729 ("most purification procedures result in considerable proteolytic activation and/or degradation of factor IX").

[14] Choi et al., Inhalation delivery of proteins from ethanol suspensions, Applied Biological Sciences 98(20): 11103-11107 (2001) ("protein powders . . are liable to clump formation").

K. BURDEN IMPROPERLY SHIFTED

Examiner has improperly shifted the burden to Applicants to prove that two non-identical formulations are not the same.[15] This is incorrect because to establish an inherent property, Examiner must **first** show that the property is **necessarily** present. *In re King*, 801 F.2d 1324 1327 (Fed. Cir. 1986) (**after** the PTO establishes a *prima facie* case of anticipation based on inherency, the burden shifts to appellant to 'prove that the subject matter shown to be in the prior art does not possess the characteristic relied on.'").[16]

L. COMPETENT EVIDENCE NOT PROVIDED

The Examiner failed on repeated request to provide a Declaration under penalty of law that the Lechuga formulation possesses **all** properties identical to those claimed. Therefore, Examiner's speculation as to the similarity of the formulations is mere argument, **not** fact. *Fiers v. Revel*, 984 F.2d 1164 (Fed. Cir. 1993) (holding that "the Board did not err in determining that Fiers presented no convincing evidence" where applicant only showed "argument ... 'unsupported by competent evidence, entitled to little or no weight . . .'"); *In re Juillard*, 476 F.2d 1380 (C.C.P.A.) ("arguments cannot take the place of evidence"); *Brand v. Miller*, 487 F.3d 862, 868-69 (Fed. Cir. 2007) ("findings of fact by the Board **must in all cases** be supported by **substantial evidence** in the record . . . in the context of a contested case, it is **impermissible** for the Board to base its factual findings on its expertise, rather than on **evidence** in the record.") (citation omitted); *Enzo Biochem v. Gen-Probe, Inc.*, 424 F.3d 1276, 1238 (Fed. Cir. 2005) (stating in summary judgment context that "**mere arguments** . . . are **insufficient** to **satisfy its burden of coming forward with evidence**") (emphasis added).

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[15] Additionally, it is not possible to recreate the powder of Lechuga because the methodology is not provided.

[16] *See also, In re Simpson,* 102 Fed. Appx. 675, 678 (Fed. Cir. 2004) (non-precedential) ("the existence of the **same** structural elements in Rilitz gave both the examiner and the Board a reason to believe that these elements could perform the same functions claimed by Simpson. The burden therefore shifted to Simpson to disprove inherency."). Here, the formulations are **not** the same, and the burden cannot be prematurely shifted.

Applicants challenge all assumptions as not properly Officially Noticed, nor supported by Declaration as **required** by 37 CFR 1.104(d)(2)[17] and under *Brand v. Miller* and other cited Federal Circuit cases. See also MPEP 2144.03.[18]

M.OBVIOUSNESS CASE NOT PROPERLY BASED ON UNKNOWN INHERENCY

Even if the formulations were identical, it is not proper to make an obviousness case based on **unknown** inherent properties of the Lechuga formulation. *In re Rijckaert,* 9 F.3d 1531, 1534 (Fed. Cir. 1993) ("That which may be inherent is not necessarily known. **Obviousness cannot be predicated on what is unknown.**") (citations omitted).

It was not known under Lechuga that any formulation of FIX could be sequestered and thus produce at least 100 hrs of dosing. It could not be predicted from Lechuga because Lechuga never used an aerosol to treat an animal or patient, much less to produce the sequestration effect allowing for less frequent dosing. The prior art pharmacokinetics also teach away—e.g., that FIX is rapidly cleared.

The dog study performed by inventors and shown in Example 1 of the specification shows proof of concept—that FIX can be absorbed when a liquid FIX is deposited on the back of the throat. However, it does not show FIX deposition in the deep lung, nor sequestration, nor 100 hour dosing.

Assuming both sequestration and 100 hour dosing is a clear application of **hindsight** reasoning. *KSR Int'l Co.* at 1742 ("A factfinder should be aware, of course, of the distortion caused by hindsight bias and must be cautious of arguments reliant upon ex post reasoning."). As such, and without any support, these assumptions are both **arbitrary and capricious**.

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[17] *Id.* ("When a rejection in an application is based on facts within the personal knowledge of an employee of the Office, the data shall be as specific as possible, and the reference **must be supported, when called for by the applicant, by the affidavit of such employee**, and such affidavit shall be subject to contradiction or explanation by the affidavits of the applicant and other persons.").

[18] *Id.* ("It is never appropriate to rely solely on 'common knowledge' in the art without evidentiary support in the record, as the principal evidence upon which a rejection was based.").

N. PRIOR ART TREATMENTS TEACH AWAY

Examiner states that "Examiner does not understand Applicants arguments as to why the difference between intravenous administration of Factor IX is relevant to overcoming the *prima facie* obviousness case."[19]

The **only** treatment for hemophiliacs available today is intravenous based treatment. Thus, this is the **closest** prior art relating to the claimed treatment method and it clearly teaches **away** from both sequestration and 100 hour dosing (See Figure 8).[20]

O. ART TEACHES AWAY

Examiner states that "the problems associated with the nebulization of aqueous solutions of proteins (e.g. Factor IX) are irrelevant with regards to the suggested method of Lechuga." However, Gupta[21] is the **only** prior art effort to aerosolize FIX **and** determine its subsequent activity. Gupta is relevant because it is the **closest** prior art related to the recited activity element (at least 80%). Yet Gupta teaches that the FIX was denatured when aerosolized. Even though Gupta teaches activity loss in liquid FIX, the formulation made by the inventors is also liquid prior to being dried, and the potential for loss of activity is similarly present. Indeed, many protein treatments are known to cause activity loss,[22] and based on this and the teachings of

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[19] Office Action, 10/26/2007, p. 8.

[20] *See, e.g.*, BeneFix package inserts at http://www.wyeth.com/content/ShowLabeling.asp?id=92 or http://www.fda.gov/cber/label/cfixwye071106lb.pdf showing 18.8 ± 5.4 or 20.2 ± 4.0 hour half lives and recommending 12-24 hour dosing. See also Fig. 8 in the specification showing **head-to-head** comparison of pharmacokinetics of IV versus inhaled FIX (Exhibit 1).

[21] Lechuga fails to demonstrate the activity or form of spray dried FIX—thus, Lechuga also fails to establish a reasonable expectation of success.

[22] *See e.g.*, Gupta, *et al.*, Pulmonary Delivery of Human Protein C and Factor IX Oxygen Transport to Tissue XVIII, Chapter 55, p. 429-435 (1997) ("Formulation of proteins into MDI's [metered dose inhalers] and DPI's [dry powder inhalers] is a **formidable** task because dehydration and subsequent communition of proteins to produce powders in the size range suitable for inhalation may lead to **loss of activity**.") (emphasis added); Huang, *et al.*, US6280729 ("most purification procedures result in considerable proteolytic activation and/or **degradation of factor IX**") (Exhibits 2 and 4).

Gupta, it is arbitrary and capricious to **assume** protein activity in the **absence of any proof** of same.

P. UNEXPECTED RESULTS FOUND IN SEQUESTRATION

Even if the *prima facie* case were made, Applicants have provided competent evidence of unexpected results in the sequestration effect (see Figure 8 showing IV profile with very high initial dose and rapid loss, as well as inhaled profile with dose remaining constant for at least 100 hrs). *See also* BeneFix package insert confirming the rapid clearance of FIX in the prior art.[23]

The pharmacokinetic profile of inhaled FIX is a significant (and surprising) improvement over the intravenous FIX profile because it **avoids the large initial dose** and thus clotting difficulties due to the initial high dose of FIX (see e.g., BeneFix package insert[24] noting that "use of factor IX complex concentrates has historically been associated with the development of thromboembolic complications")[25], and because the **dose remains constant** for at least 100 hours. Further, based on the surprisingly flat pharmacokinetics shown in Figure 8, one would expect that the FIX would remain sufficiently high to prevent excess bleeding for at least one week. Thus, the clinical importance of this unexpected effect is also shown. This unexpected (and claimed) effect is sufficient to rebut a *prima facie* case of obviousness.

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[23] See e.g., http://www.wyeth.com/content/ShowLabeling.asp?id=92 or http://www.fda.gov/cber/label/cfixwye071106lb.pdf (two slightly different BeneFix package inserts providing half lives of 18.8 ± 5.4 hours or 20.2 ±4.0 hours).

[24] E.g., http://www.wyeth.com/content/ShowLabeling.asp?id=92.

[25] *See also*, Astrid van Hylckama Vlieg, *et al.*, **High levels of factor IX increase the risk of venous thrombosis,** Blood, 95(12):3678-3682 (2000) (emphasis added); Huang, et al. US6280729 ("Oversupply of one or more substances, particularly activated coagulation factors, may lead to **unwanted coagulation**. . . There are numerous reports in the literature on the adverse clinical consequences of administering prothrombin complex concentrate (or other factor IX concentrates) contaminated with factor IXa and/or with active or degraded forms of other clotting factors. The most serious risk is the inadvertent activation of the clotting cascade. **Deaths** have been documented.") (emphasis added) (see Exhibit 4).

Q. EXAMINER SUMMARILY DISMISSES HEAD-TO-HEAD DATA

Examiner summarily dismisses the evidence of unexpected results as "off point" since its relates to injection and not inhalation treatments. However, the BeneFix package inserts showing an 18-20 hr half life **is the closest prior art relating to the treatment element**, and Examiner has not cited **any** other prior art showing treatment.[26] It is procedurally incorrect to summarily dismiss all prior art that unfortunately teaches away from the invention as "off point."

Further, the pharmacokinetic data is reproduced by inventors in a head-to-head measurement of IV versus inhalation pharmacokinetics—data even the FDA would accept in making a direct drug comparison claim. There is no rationale basis for ignoring this data, and its summary dismissal is both **arbitrary and capricious**.

R. PRIOR ART AND *IN VIVO* DATA PROVIDE COMPETENT EVIDENCE

Applicants have shown **published statements** by Gupta and others in the field evidencing no reasonable expectation of success and ***in vivo* head-to-head comparative data** showing unexpected effects, thus rebutting the *prima facie* case. Competent rebuttal evidence taken as a whole should be weighed against the evidence supporting the *prima facie* case. *In re Piasecki*, 745 F.2d 1468, 1472 (Fed. Cir. 1984). See also MPEP 716.01(d) ("All of the **competent** rebuttal evidence . . . should be weighed") (emphasis added).

In this case, there is **no** competent evidence for the opposing case, which is based **only on argument and assumption** by the Examiner.

Thus, as a **matter of law** the unsupported rejections should be withdrawn. *Brand v. Miller*, 487 F.3d 862, 868-69 (Fed. Cir. 2007) ("findings of fact by the Board must in all cases be supported by **substantial evidence** in the record . . . in the context of a contested case, it is **impermissible** for the Board to base its factual findings on its expertise, rather than on **evidence** in the record.") (citation omitted); *In re Pearson,* 494 F.2d 1399, 1405 (CCPA 1974 ("The fatal

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[26] Lechuga does **not** show inhalation treatment with FIX, but only states what was already known in the art—that FIX could be used to treat hemophilia.

defect in this argument is that there is no competent evidence . . . argument in a brief cannot take the place of evidence.") (citation omitted); *Truswal Systems Corp. v. Hydro-Air Engineering, Inc.*, 813 F.2d 1207, 1211 (Fed. Cir. 1987) ("We reject the unsupported statements . . .").

8. CONCLUSION

The Examiner rejects claims 29, 32-33, 36-37 and 40 as obvious BUT has failed to provide **any** support for **two** of the recited claim elements, and has **assumed** the existence of another **five elements** based on an prior art formulation that is **not even identical** to that taught in the cited art. Further, the art teaches that the field is unpredictable, and the only prior attempt to make aerosolized FIX was a failure. Thus, seven elements are missing from the cited art and there is no reasonable expectation of success. Thus, the *prima facie* case is not made, and the rejection should be withdrawn for claims 29, 32-33, 36-37 and 40.

Further, no art shows the detailed preparation parameters recited in dependent claims 32 and 36, and the art cited to support the obviousness of such claims actually teaches away since the diafiltration method resulted in only 10% FIX. Thus, even if the basic claims were obvious (and they are not) the dependent claims are still allowable, and the rejection should be withdrawn for at least these claims.

Finally, Applicants have provided evidence of non-obviousness (teaching away in the prior art and *in vivo* data showing an unexpected pharmacokinetic result) sufficient to rebut a *prima facie* case for all pending claims. Thus, as a matter of law claims 29, 32-33, 36-37 and 40 cannot be held to be obvious and Applicants request their allowance.

If any questions or issues remain in the resolution of which the Board feels will be advanced by a conference with the Applicants' attorney, the Board is invited to contact the attorney at the number noted below. The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account No. 50-3420 (reference 31176282-004001 Valoir).

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Respectfully submitted,

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APPENDIX A: CLEAN COPY OF CURRENT PENDING CLAIMS

Claim 29 (previously presented) A method of preventing hemophilic bleeding in advance of a bleeding event, said method comprising:

a) aerosolizing a monomeric Factor IX (FIX), wherein the aerosolized monomeric FIX: i) has a mass median aerodynamic diameter (MMAD) of between 2 and 4 μm, ii) has a fine particle fraction percent less than 3.3 μm (FPF %<3.3 μm) of at least 50%, iii) is at least 90% monomeric, iv) wherein the after-aerosolization activity/pre-aerosolization activity is at least 80%; and v) is a dry powder having less than 10% water (wt/wt), but does not have ethanol;

b) slowly maximally inhaling aerosolized monomeric FIX; and

c) allowing said monomeric FIX to deposit in the deep lung tissue such that said monomeric FIX is sequestered in said deep lung tissue to provide sufficient FIX to prevent bleeding for at least 100 hours after administration.

Claim 32 (previously presented) The method of claim 29, wherein said FIX is prepared by

a) diafiltering concentrated FIX solution to a concentration of approximately 12 mg/ml;

b) spray drying the diafiltered solution at 40 or 60 psi and 60°C or 70°C at 5 ml/min and 17.8 standard cubic feet per minute (scfm); and

c) transferring spray dried FIX to a sealed storage container at less than 5% relative humidity.

Claim 33 (previously presented) A prophylactic method of treating hemophilia, said method comprising

a) aerosolizing a monomeric Factor IX (FIX), wherein the aerosolized monomeric FIX: i) has a mass median aerodynamic diameter (MMAD) of between 2 and 4 μm, ii) has a fine particle fraction percent less than 3.3 μm (FPF %<3.3 μm) of at least 50%, iii) is at least 90% monomeric, iv) wherein the after-aerosolization activity/pre-aerosolization activity is at least 80%; and v) is a dry powder having less than 10% water (wt/wt), but does not have ethanol;

b) slowly maximally inhaling aerosolized monomeric FIX;

c) allowing said monomeric FIX to deposit in the deep lung tissue, and

d) followed by exhalation, wherein said monomeric FIX is sequestered in said deep lung tissue to provide sufficient FIX to prevent bleeding for at least 100 hours after administration.

Claim 36 (currently amended) The method of claim 33, wherein said FIX is prepared by

a) diafiltering concentrated FIX solution to a concentration of approximately 12 mg/ml;

b) spray drying the diafiltered solution at 40 or 60 psi and 60°C or 70°C at 5 ml/min and 17.8 standard cubic feet per minute (scfm); and

c) transferring spray dried FIX to a sealed storage container at less than 5% relative humidity.

Claim 37 (previously presented) A method of preventing hemophilic bleeding in advance of a hemophilic assault, said method comprising:

a) aerosolizing a Factor IX (FIX), wherein the aerosolized FIX: i) has a mass median aerodynamic diameter (MMAD) of between 2 and 4 μm, ii) has a fine particle fraction percent less than 3.3 μm (FPF %<3.3 μm) of at least 50%, iii) is at least 90% monomeric, iv) wherein the after-aerosolization activity/pre-aerosolization activity is at least 80%; and v) is a dry powder having less than 10% water (wt/wt);

b) inhaling the aerosolized FIX at least once per week and allowing the aerosolized FIX to deposit in the lung; and

c) followed by exhalation wherein said monomeric FIX is sequestered in said lung tissue to provide sufficient FIX to prevent bleeding for at least 100 hours after administration.

Claim 40 (previously presented) A prophylactic method of treating hemophilic bleeding, said method comprising:

a) aerosolizing a Factor IX (FIX), wherein the aerosolized FIX: i) has a mass median aerodynamic diameter (MMAD) of between 2 and 4 μm, ii) has a fine particle fraction percent less than 3.3 μm (FPF %<3.3 μm) of at least 50%, iii) is at least 90% monomeric, iv) wherein the after-aerosolization activity/pre-aerosolization activity is at least 80%; and v) is a dry powder having less than 10% water (wt/wt);

b) slowly maximally inhaling aerosolized monomeric FIX; and

c) allowing said monomeric FIX to deposit in the lung such that said monomeric FIX is sequestered in said lung to provide sufficient FIX to prevent bleeding for at least 100 hours after administration.

APPENDIX B: EVIDENCE

Exhibit 1: Figure 8, filed as part of the specification on April 8, 2004.

Exhibit 2: Gupta, *et al.*, Pulmonary Delivery of Human Protein C and Factor IX Oxygen Transport to Tissue XVIII, Chapter 55, p. 429-435 (1997), cited with IDS on 7/20/04, and entered by Examiner on 2/12/06.

Exhibit 3: Lechuga, *et al.*, WO0132144, cited with Supplemental IDS on 5/16/05, and entered by Examiner on 2/12/06.

Exhibit 4: Huang, *et al.*, US6280729, cited by Examiner on 4/13/2007.

Exhibit 5:Kurachi, *et al.*, Biology of Bactor IX, Blood Coagulation and Fibrinolysis, 4: 953-974 (1993), cited by Examiner on 7/27/2006.

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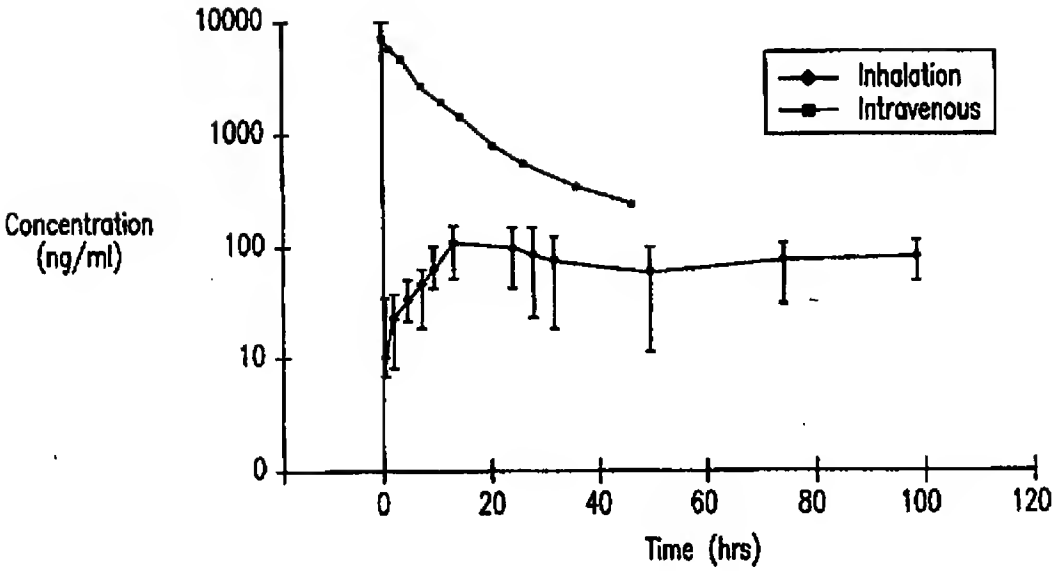

Mean Corrected Concentration Time Curve for Tolerized Hemophilia B Dogs (n=3) Receiving rF.IX (50 IU/kg) by Inhalation

FIG. 8

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PULMONARY DELIVERY OF HUMAN PROTEIN C AND FACTOR IX

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1. INTRODUCTION

Recent advances in structural elucidation of numerous natural peptides and proteins[1], enhanced understanding of their role in several physiological processes, and the use of biotechnological techniques for their production have stimulated considerable interest in establishing peptides and proteins as therapeutic agents. A major problem, aside from proteolytic degradation and physical alteration of the protein molecule at the site of administration, is the slow rate of transport of macromolecules across membrane barriers into the systemic circulation.

Inhalation offers some exciting possibilities, since the route offers relatively short pathlength (0.4–1.0 μm) between the pulmonary epithelium and circulation coupled with the extensive absorptive surface and smaller airways of the alveoli (70 m^2 in man). This route of delivery has proven capable of delivering even large macromolecules with acceptable bioavailabilities. The objectives of this work were:

A) To Show that Aerosolization Can Potentially Destabilize Proteins. During jet nebulization denaturation may occur due to shear forces associated with the production of

Oxygen Transport to Tissue XVIII, edited by Nemoto and LaManna
Plenum Press, New York, 1997

small droplets or the large air-water interface continuously being produced within the nebulizer[3]. Oxidation of proteins usually plays a minor role in overall deactivation.

B) To Demonstrate the Feasibility of Protein Delivery through the Lungs. A large alveolar surface area and a thin diffusion barrier makes the lungs a viable route of drug delivery for macromolecules such as Protein C and Factor IX. It avoids first pass hepatic metabolism while minimizing the opportunities for pulmonary metabolism due to relatively short residence time.

C) To Study a Delivery System that Can Serve as a Model for Delivery of Other Vitamin K Dependent Clotting Factors of Therapeutic Use. There are seven vitamin K dependent coagulation proteins (factors VII, IX, X and prothrombin and anticoagulant proteins, protein C and protein S) with similar structures (amino acid homology and molecular architecture) and physical properties. Information gained with Factor IX and Protein C may be applicable with little or no modification to the others.

1.1. Protein C and Factor IX as Model Proteins

These plasma proteins are produced by hepatocytes and need vitamin K for biosynthesis. They circulate in the blood in their inactive forms and are converted to active enzymes when blood coagulation is initiated. Factor IX (Christmas factor) is a single chain glycoprotein with a molecular weight of 56,800 kDa[3]. It occurs in blood plasma at concentrations of 4 μg/ml and has a half life ($t_{1/2}$) of 22 hrs. Hemophilia B patients are currently treated periodically with IV injections of Factor IX to control bleeding episodes. Episodic treatment is ineffective in preventing bleeding into the joints which results in joint degeneration and painful crippling deformities. A less invasive dosage regimen would enable the prophylactic maintenance of homeostatic control, which would prevent joint degeneration and greatly enhance the quality of life for the hemophilia B patient.

Protein C, unlike Factor IX is an anticoagulant and circulates as two polypeptide chains with an average molecular weight of 62 kDa. It occurs in blood plasma at 4 μg/ml and has a much shorter half life ($t_{1/2}$= 6–8 hrs) than Factor IX. In clinical situations, such as deep vein thrombosis (clot formation in vein), or pulmonary embolism, where a thrombus has already formed, rapid activation of the fibrinolytic systems by administration of anticoagulants is effective in diminishing the thrombus. The two major types of clinically useful anticoagulants, heparins and coumarins are associated with undesirable effects including thrombocytopenia, drug-drug interactions, bleeding, and skin necrosis[4]. Administration of a naturally occurring anticoagulant, like Protein C would potentially solve these problems while being therapeutically advantageous to the patient.

2. DELIVERY OPTIONS AND CONCERNS

Currently available options for the generation of inhalable aerosols include various types of nebulizers, propellant based systems (metered dose inhalers) and dry powder inhalers (DPI's). The critical issues in lung delivery of therapeutic peptides and proteins are shown in Table 1.

Formulation of proteins into MDI's and DPI's is a formidable task because dehydration and subsequent communition of proteins to produce powders in the size range suitable for inhalation may lead to loss of activity. In contrast there are numerous proteins

Table 1. Primary concerns during aerosol generation

1.	Drug denaturation/Inactivation including aggregation and adsorption to device
2.	Dispersibility(surface energetics, surfactant compatibility, propellant type)
3.	Lung deposition efficiency(dose, particle size distribution)
4.	Patient compliance, correlation of pharmacokinetic and pharmacodynamic data
5.	Formulation issues and environmental impact of MDI propellants
6.	Dose delivery efficiency and accuracy.

which can be nebulized, since they are invariably purified and initially formulated in aqueous solutions for parenteral administration. The air-blast nebulizer in its simplest form uses the Bernouilli effect to draw liquid up a concentric supply capillary that surrounds a narrow jet supplied with compressed air. More than 99% of the nebulized fluid is recirculated following impaction on internal baffles while only a small fraction of the aerosol escapes as inhalable aerosol.[5] Increased recycling promotes protein degradation. In this work we administered Factor IX using two types of nebulizers, a Side Stream disposable and the Pari LC jet Plus. In order for the protein to be absorbed systemically, the administration technique must maximize drug deposition in the pulmonary region. Stable aerosol systems with mass median aerodynamic diameters (MMAD) of less than 5 μm deposit about 70% of their discharged dose in the respiratory tract following slow oral inhalation.[6] Nebulized aqueous solutions, which deliver from 0% to 20% of their initial drug load are similar in efficiency to MDI's. Penetration of peripheral airways as opposed to tracheobronchial deposition appears to be more effective by nebulization of aqueous solutions, rather than with MDI's or Dry powder inhalers.

3. MATERIALS AND METHODS

Due to the high costs and limited availability of purified Protein C, our preliminary experiments were carried out using highly purified Factor IX . Purified human Factor IX was provided by the American Red Cross. Hence, the experimental procedures have been established with Factor IX, and experiments will be repeated with Protein C.

ELISA's were performed in Immunolon II, 96 well, flat bottom microtiter plates from Dynatek. Capture buffer (0.1M sodium bicarbonate, 1.0M sodium chloride, pH= 9.6), blocking buffer (0.05M monosodium phosphate, 0.1M sodium chloride, 10mg/ml BSA pH= 7.3) and wash buffer (0.05M monosodium phosphate, 0.1M sodium chloride, 0.05% Tween-20) are the three buffers used for Factor IX polyclonal ELISA procedure.

Capture antibody (Dako A300 Rabbit Anti-human Factor IX) used to coat the plates overnight, detection antibody (Boehringer mouse anti-human Factor IX) and labeled antibody (Dako P161 Rabbit anti-mouse IgG, HRP Labeled) were the three antibodies used for the Factor IX ELISA. NBS two component peroxidase from KPL labs was used as the substrate for the antibodies.

In order to evaluate the Factor IX activity a clotting assay was used. Congenital Factor IX deficient human plasma from universal reagents was mixed with the samples along with KONTACT APTT from pacific Hemostasis. Only active Factor IX reacts with the other clotting agents in the deficient plasma to form a clot. The rate of clot formation would suggest the amount of Factor IX active in our samples. Lancer Coagulyzer from CMS was used to study the clot times. The samples were diluted in BAT buffer (0.05M Imidazole, 0.1M NaCl, 0.1% (w/v) BSA AND 0.01% Tween-20).

3.1. Nebulizer Dryer Apparatus

The unit has four main components; air supply, heating system, nebulizer, and drying chamber. Fig. 1 shows a schematic of the apparatus. A nylon tube carries an 80 psig air supply to a pressure regulator. The air supply is split into two branches, each connected via a rotameter (Variable area flowmeters, 65 mm, Cole Parmer Instruments.) One branch directs air to a heating system. The second branch supplies pressurized air to the nebulizer. The air heating system consists of a fifty foot, one-half inch diameter, coiled copper tube wrapped with 2" x 8' silicone rubber heating tape (Barnstead/Thermolyne) connected to a proportional controller (Type 45500 Input Controller, Barnstead/Thermolyne). A nebulizer (Side stream disposable nebulizer or Pari LC Jet Plus nebulizer) generates droplets directly into the left side of the drying chamber via a "T" connector. One limb of the connector is blocked during experiments to drive all the aerosol into the drying chamber. As shown in the figure, the airtight drying chamber consists of a plexiglass cylinder 12 inches in height, with an internal diameter of 7 inches. The cylinder is divided vertically by a 0.25 inch plexiglass baffle to within 0.05 inch of the top. Drying air is fed into the bottom of the left side after passing through an expansion chamber approximately 1.5 inches in height and 3 inches in diameter. The nebulizer is attached through an orifice 3.5 inches above the base in the left side of the drying chamber. The copper exit tubing is located on the right side of the chamber at the base. This serves as an outlet for respirable size aerosol particles, which are bubbled into a buffer with a sparger or connected into a rat exposure chamber. Droplets exiting the nebulizer travel up the left side of the baffle, across, then down the right side of the drying chamber, carried by the drying air stream, before reaching the copper exit tube.

Advantages of this geometry include the impaction of excessively large droplets exiting the nebulizer on the central baffle, resulting in their removal from the product stream, while rapidly sedimenting droplets do not immediately contact a surface, and can thus be dried and reduced in size allowing them to remain entrained in the product stream.

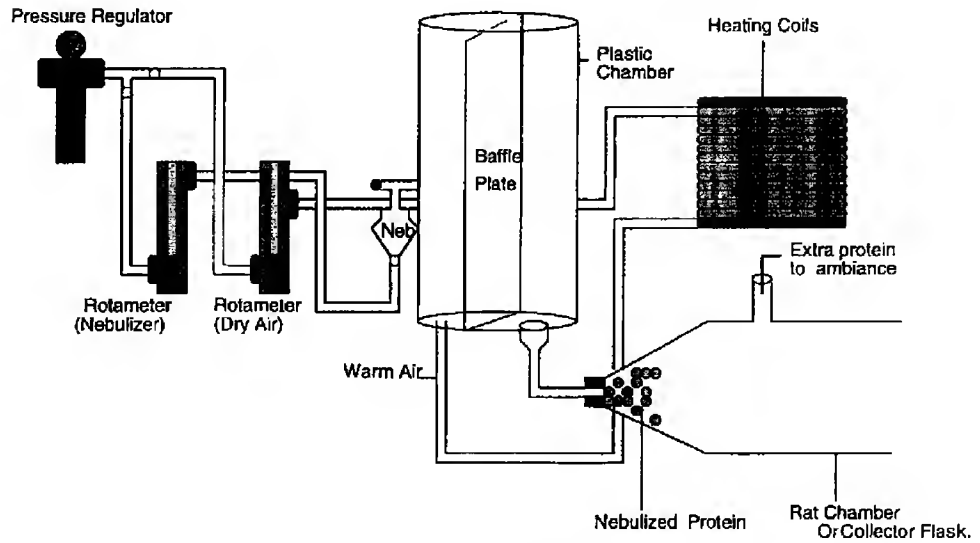

Figure 1. Equipment set up.

The drying chamber has a volume of 10.6 liters. leading to mean residence time 10.6 seconds at air flows of 60 liters/minute. Increased residence times (lower flow rates) lead to more complete drying and a higher concentration of inhalable aerosol particles (2–5 μm diameter), so long as the air stream does not become saturated with water. The experimental conditions for Factor IX delivery are given in Table 2.

4. RESULTS AND DISCUSSION

The data provided in this paper are for Factor IX. The in vitro experiments were performed using the Side Stream and the Pari LC jet plus nebulizers. The primary difference between the two is in their baffle design. Pari LC is a high output nebulizer producing more aerosol particles in the respirable range (2–5 μm diameter).

The objectives of the experiment were defined in terms of the following parameters:

- $\eta 1$= Fraction of the protein aerosolized.
- $\eta 2$= Fraction of the protein active after aerosolization.
- $\eta 3$= Fraction of the protein still active after exposure to nebulizer shear stress.

The primary objective was to maximize the amount of protein delivered from the nebulizer without deactivating it. Therefore the system efficiency (η_{sys}) could be expressed as:

$$\eta_{sys} = \eta_1 * \eta_2$$

Initial experiments showed no protein collection due to failure of the impact sparger to the aerosol. To solve this problem we added a vacuum pump drawing 60 L/min additional make-up air to accelerate the particles and compare the collection efficiency. With this modification 19.8% of the aerosolized Factor IX was collected. Our objective was to collect respirable (2–5 μm) size particles using the drying chamber to provide enough residence time to reduce droplet size of the aerosol to the respirable range. Hence, the 19.8% efficiency represents a good estimate of respirable dose produced by the nebulizer. Unfortunately of this 20% none was active after collection. We hypothesized that in the process of aerosolization Factor IX was denatured due to shear forces imposed by the nebulizer or the large air water interface produced during the nebulization.

Table 2. Experimental conditions for Factor IX delivery

Compressed air pressure	50 psig.
Factor IX concentration in the nebulizers	40 μg/ml
Dry air flow rate to the chamber	10-12 liters/min.
Air flow rate to the chamber	3-5 liters/min.
Vacuum pump suction rate	60 liters/min.
Temperature in the drying chamber	45^0 C
Side stream nebulizer dose	10 ml or 400 μg/ml.
Side stream dosing time	75 mins.
Pari LC dose	8ml or 320 μg/ml.
Pari LC dosing time	15 mins.

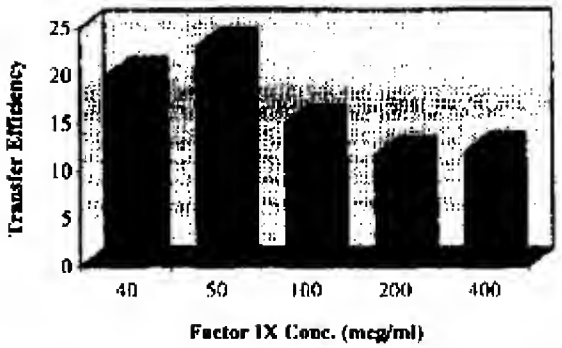

Figure 2. Optimal dose evaluation for Factor IX. Nebulizer flow rate: 3.4 L/min, compressure pressure: 50 psig.

To prevent protein denaturation bovine serum albumin (BSA) at 15 mg/ml, representing almost 400 times the Factor IX concentration was added. The proteins are surface active and tend to migrate to an air water interface. Their migration rate to the interface depends on their concentration in the formulation. Adding BSA at a much greater concentration not only helps in shielding the protein from air-water interface denaturation but also protects it from nebulizer shear stresses. Two control parameters, protein activity or nebulizer output were identified. Further improvement of the protein activity requires a thorough study of the protein's chemistry, which was considered a more formidable task than using a higher output nebulizer. The results clearly show that the system efficiency (η_{sys}) was doubled with the Pari LC jet nebulizer compared to the Side Stream nebulizer. Pari nebulizer also imposed more severe shear stresses on the protein and therefore denatured the protein by 35% showing itself to be more efficient in production of respirable size particles, at the expense of compromised protein stability. Therefore, a thorough study of protein deactivation due to device shear stresses is important while developing a potential protein aerosol delivery system.

Table 3. *In vitro* results for aerosol delivery of Factor IX

Experiment	Result	Conclusion
Factor IX solution (40µg/ml) nebulized in the Side Stream nebulizer	No protein was collected in the sparger collection flask. η_1= 0%.	Sparger flow rate increased to 60 L/min using a vacuum pump. η_{sys} = 0%.
Factor IX solution (40 µg/ml) was again nebulized with the new set up.	Protein was collected completely deactivated. η_1= 21%, η_2= 0%, η_3=100%	Large air-water interface denaturation. BSA added at 15 mgs/ml. η_{sys}= 0%
Factor IX (40µg/ml) nebulized in the side stream nebulizer with the new formulation.(Factor IX + BSA)	η_1= 19.8%. η_2= 53% η_3 = 100%	η_{sys}= η_1*η_2 = 10.5%10% of Factor IX can be effectively delivered.
Factor IX nebulized in the Pari LC Jet plus nebulizer with the new formulation(Factor IX + BSA)	η_1= 40%. η_2= 50% η_3= 65%	η_{sys}= η_1*η_2 = 20%This nebulizer has doubled the efficiency.

When attempting to maximize drug delivery, high protein concentration might not always lead to optimal results. Evaporation of droplets leads to increased protein concentration within the nebulizer reservoir and may ultimately destabilize the macromolecules depending on the rate of protein aggregation. At concentrations above the optimal value nebulizers may produce reduced respirable dose and thereby compromise on the nebulizer transfer efficiency. The plot below shows that 50 μg/ml is the optimal Factor IX concentration when using the following delivery parameters under the conditions indicated in the legend.

CONCLUSIONS

In the process of being aerosolized human Factor IX is 50% denatured at the air water interface. Pulmonary delivery optimization of proteins is a function of two parameters, device transfer efficiency and protein activity. An optimization of these control parameters is essential to develop a potential protein delivery system. Using Pari rather than the sidestream doubled the nebulization efficiency (η_1) but denatured 35% of the protein due to nebulizer shearing. The Side Stream nebulizer did not denature the protein due to shearing but generates a lower output of respirable particles. Thus high output nebulizers can cause denaturation of the protein due to their shearing. Bovine Serum Albumin (BSA) plays an important role in protecting the protein from interface deactivation. In vivo Factor IX delivery in rats to study the bioavailability and characterize its absorption from the lungs is in progress. On the basis of these results we shall then study the feasibility of Protein C delivery.

ACKNOWLEDGMENTS

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REFERENCES

1. Vincent H. Lee: Changing needs in drug delivery in the era of peptide and protein drugs. *Peptide and Protein Drug Delivery*. Advances in Parenteral Sciences/4. (1991) 1–57.
2. Peter R. Byron: Determinants of drug and polypeptide bioavailability from aerosols delivered to the lung. *Advanced Drug Delivery Reviews*. 5 (1990) 107–132.
3. Ulla Hedner, Earl W. Davie: Introduction to Hemostasis and vitamin K dependent coagulation factors.. *Blood and blood forming tissue*. 2107–2133
4. J. Bryan Smith: Drugs affecting coagulation, fibrinolysis, and platelet aggregation. *Human Pharmacology. Molecular to Clinical*. (1995) 21.
5. Ralph W. Niven: Delivery of Biotherapeutics by Inhalation aerosols. Ralph W. Niven. *J. Pharmaceutical Technology* (July 1993) 72.
6. Peter R. Byron :Pulmonary targeting with aerosols. *J. Pharmaceutical Technology* (43). May 1987.

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(54) Title: DRY POWDER COMPOSITIONS HAVING IMPROVED DISPERSIVITY

(57) Abstract: The present invention provides a highly dispersible formulation comprising an active agent and a dipeptide or tripeptide comprising at least two leucyl residues. The composition of the invention possesses superior aerosol properties and is thus preferred for aerosolized administration to the lung. Also provided are a method for (i) increasing the dispersibility of an active-agent containing formulation for administration to the lung, and (ii) delivery of the composition to the lungs of a subject.

DRY POWDER COMPOSITIONS HAVING IMPROVED DISPERSIVITY

This application claims the benefit of priority of the following U.S. Provisional Patent Applications: Serial No. 60/162,451 filed on October 29, 1999; Serial No. 60/164,236 filed on November 8, 1999, Serial No. 60/172,769 filed on December 20, 1999; Serial No. 60/178,383 filed on January 27, 2000; and Serial No. 60/178,415 filed on January 27, 2000, all of which are incorporated herein by reference in their entirety.

Field of the Invention

The present invention is directed to highly dispersive dry powder compositions, and in particular, to highly dispersive, inhalable dry powder compositions for aerosolized delivery to the lungs. The dry powders of the invention contain an active agent and a di- or tripeptide containing at least 2 leucyl residues, and are physically and chemically stable upon storage. The powders of the invention also demonstrate superior aerosol performance.

Background of the Invention

Traditionally, inhalation therapy has played a relatively minor role in the administration of biotherapeutics and conventional pharmaceuticals when compared to more traditional drug administration routes, such as oral and intraveneous. Injection is the customary route of delivery of biotherapeutics (e.g., peptides, proteins and nucleic acids), and due to the many drawbacks associated with injection (e.g., inconvenience, discomfort, patient aversion to needle-based delivery methods), alternative administration routes are needed.

Pulmonary delivery is one such alternative administration route which can offer several advantages over subcutaneous administration. These advantages include the convenience of patient self-administration, the potential for reduced drug side-effects, ease of delivery by inhalation, the elimination of needles, and the like. Many preclinical and clinical studies with inhaled proteins, peptides, DNA and small molecules have demonstrated that efficacy can be achieved both within the lungs and

systemically. However, despite such results, the role of inhalation therapy in the health care field has not grown as expected over recent years, in part due to a set of problems unique to the development of inhalable drug formulations. Dry powder formulations, while offering unique advantages over cumbersome liquid dosage forms and propellant-driven formulations, are prone to aggregation and low flowability phenomena which considerably diminish the efficiency of dry powder-based inhalation therapies.

Particulate aggregation, caused by particle-particle interactions, such as hydrophobic, electrostatic, and capillary interactions, must be minimized in order to provide dispersible powders for effective inhalation therapies. Various approaches have been utilized in efforts to prepare dry powders having minimal particle aggregation and good aerosol properties. These approaches include the modification of dry powder particle surface texture (Ganderton, et al., U.S. Pat. No. 5,376,386), the co-delivery of large carrier particles (absent drug) with therapeutic aerosols to achieve efficient aerosolization, particle coatings (Hanes, 5,855,913; Ruel, et al., 5,663,198) aerodynamically light particles (Edwards, et al., 5,985,309), use of antistatic agents, (Simpkin, et al., 5,908,639) and the addition of certain excipients, e.g., surfactants (Hanes 5,855,913; Edwards, 5,985,309). Unfortunately, the formation of particulate aggregates and production of powders having poor flow properties and low dispersivities continue to plague development efforts to prepare aerosolizable dry powders for inhalation therapy. Thus, a need exists for improved inhalable aerosols for the pulmonary delivery of therapeutic agents, and in particular, for dry powders having excellent aerosol properties and reduced particle-particle interactions, irrespective of the therapeutic agent.

Summary of the Invention

The present invention is based upon the discovery of a particular class of excipients, which, when incorporated into dry powder formulations for aerosolization and delivery to the lung, notably improves the dispersivity and aerosolization properties of the dry powders, irrespective of the type of active agent contained in the formulation. More particularly, the invention provides a dry powder composition which comprises an active agent and a di or tri-peptide

comprising at least two leucines. Preferred di- and tripeptides are those which are surface active.

The dry powder of the invention typically contains from about 2% by weight to about 99% by weight di- or tri-peptide, and may optionally contain additional excipients or carriers, such as carbohydrates, amino acids, peptides, proteins, organic acid salts, and/or polymers.

The presence of the di- or tri-peptide is effective to notably increase the emitted dose of the dry powder over the emitted dose of the powder composition absent the di- or tri-peptide. In one particular embodiment of the invention, the dry powder of the invention is characterized by an emitted dose of at least about 30%. In another embodiment, the concentration of the dileucyl- di- or tri-peptide on the surface of the particles is greater than in the bulk powder.

Additional features of the dry powder particles of the invention include, in one embodiment, a mass median diameter of less than about 10 microns, and in yet another embodiment, a mass median aerodynamic diameter of less than about 10 microns. In yet another embodiment, the dry powder comprises particles having a bulk density from 0.1 to 10 grams per cubic centimeter.

The dry powder of the invention is further characterized by both physical and chemical stability upon storage, as characterized, in one embodiment, by a drop in emitted dose of no more than about 10% when stored under ambient conditions for a period of three months. In another embodiment, the chemical stability of the dry powder is characterized by degradation of less than about 5% by weight of the active agent upon storage of the dry powdered composition under ambient conditions for a period of three months.

In another aspect, the invention provides a method for enhancing the aerosol performance of a dry powder. In the method, a di- or tri-peptide is incorporated into an active-agent containing liquid formulation. The resulting liquid formulation is dried to produce a dry powder containing the active agent and the di- and/or tripeptide, whereby the resultant dry powder possesses an emitted dose that is increased over the emitted dose of a dry powder having the same components but absent the di- or tripeptide.

In one embodiment of the method, the liquid formulation is an aqueous formulation. In another particular embodiment of the method, the liquid formulation is spray-dried to produce a dry powder.

In yet a further aspect, the invention provides a method for increasing the aerosol performance of an active-agent containing formulation suitable for administration to the lung. According to the method, a di- or tripeptide comprising at least two leucines is incorporated into a formulation comprising an active agent. The resulting composition comprising the active agent and the di- or tripeptide possesses an emitted dose that is increased over the emitted dose of a composition having the same components but absent the di- or tripeptide. In one embodiment, the method results in a liquid composition suitable for aerosolized administration to the lung; in an alternative embodiment, the method results in a dry powdered composition suitable for aerosolized administration to the lung.

Yet another aspect of the invention is directed to a method for delivery of a dry powder composition to the lungs of a mammalian subject by administering by inhalation the compositions of the invention as previously described, in aerosolized form.

These and other objects and features of the invention will become more fully apparent when the following detailed description is read in conjunction with the accompanying figures and examples.

Detailed Description of the Invention

I. Definitions

The following terms as used herein have the meanings indicated.

"Active agent" as described herein includes any agent, drug, compound, composition of matter or mixture which provides some pharmacologic, often beneficial, effect that can be demonstrated *in-vivo* or *in vitro*. This includes foods, food supplements, nutrients, nutriceuticals, drugs, vaccines, antibodies, vitamins, and other beneficial agents. As used herein, these terms further include any physiologically or pharmacologically active substance that produces a localized or systemic effect in a patient.

"Amino acid" refers to any compound containing both an amino group and a carboxylic acid group. Although the amino group most commonly occurs at the position adjacent to the carboxy function, the amino group may be positioned at any location within the molecule. The amino acid may also contain additional functional groups, such as amino, thio, carboxyl, carboxamide, imidazole, etc. An amino acid may be synthetic or naturally occurring, and may be used in either its racemic or optically active (D-, or L-) form.

"Leucine", whether present as a single amino acid or as an amino acid component of a peptide, refers to the amino acid leucine, which may be a racemic mixture or in either its D- or L- form, as well as modified forms of leucine (i.e., where one or more atoms of leucine have been substituted with another atom or functional group) in which the dispersibility-enhancing effect of the modified amino acid or peptide is substantially unchanged or unimproved over that of the unmodified material.

"Dipeptide", also referred to herein as a dimer, refers to a peptide composed of two amino acids.

"Tripeptide", also referred to herein as a trimer, refers to a peptide composed of three amino acids.

A "surface active" material is one having surface activity (measured, e.g., by surface tensiometry), as characterized by its ability to reduce the surface tension of the liquid in which it is dissolved. Surface tension, which is associated with the interface between a liquid and another phase, is that property of a liquid by virtue of which the surface molecules exhibit an inward attraction.

Typically, in the context of the present invention, a surface active dipeptide or tripeptide is identified by preparing solutions of varying concentrations (from approximately 0.01% wt/vol (0.1 mg/ml) to approximately 2% wt/vol (20 mg/ml) of the subject peptide in water, and measuring the surface tension of each of the solutions. A surface-active peptide is one which, when present at any concentration in solution, though typically present in an amount greater than 0.25 mg/ml, is effective to lower the surface tension of water from its control value. A peptide that is more surface active than another peptide is one which decreases the surface

tension of water to a greater extent, when present in the liquid at the same concentration and measured under the same set of experimental conditions.

"Dry powder" refers to a powder composition that typically contains less than about 20% moisture, preferably less than 10% moisture, more preferably contains less than about 5-6% moisture, and most preferably contains less than about 3% moisture, depending upon the particular formulation.

A dry powder that is "suitable for pulmonary delivery" refers to a composition comprising solid (i.e., non-liquid) or partially solid particles that are capable of being (i) readily dispersed in/by an inhalation device and (ii) inhaled by a subject so that a portion of the particles reach the lungs to permit penetration into the alveoli. Such a powder is considered to be "respirable".

"Aerosolized" or "aerosolizable" particles are particles which, when dispensed into a gas stream by either a passive or an active inhalation device, remain suspended in the gas for an amount of time sufficient for at least a portion of the particles to be inhaled by the patient, so that a portion of the particles reaches the lungs.

"Emitted Dose" or "ED" provides an indication of the delivery of a drug formulation from a suitable inhaler device after a firing or dispersion event. More specifically, for dry powder formulations, the ED is a measure of the percentage of powder which is drawn out of a unit dose package and which exits the mouthpiece of an inhaler device. The ED is defined as the ratio of the dose delivered by an inhaler device to the nominal dose (*i.e.*, the mass of powder per unit dose placed into a suitable inhaler device prior to firing). The ED is an experimentally-determined parameter, and is typically determined using an *in-vitro* device set up which mimics patient dosing. To determine an ED value, a nominal dose of dry powder, typically in unit dose form, is placed into a suitable dry powder inhaler (such as that described in U.S. Patent No. 5,785,049, assigned to Inhale Therapeutic Systems) which is then actuated, dispersing the powder. The resulting aerosol cloud is then drawn by vacuum from the device, where it is captured on a tared filter attached to the device mouthpiece. The amount of powder that reaches the filter constitutes the emitted dose. For example, for a 5 mg dry powder-containing dosage form placed into an inhalation device, if dispersion of the powder results in the recovery of 4 mg of

powder on a tared filter as described above, then the emitted dose for the dry powder composition is: 4 mg (delivered dose)/5 mg (nominal dose) x 100 = 80%. For non-homogenous powders, ED values provide an indication of the delivery of drug from an inhaler device after firing rather than of dry powder, and are based on amount of drug rather than on total powder weight. Similarly for MDI and nebulizer dosage forms, the ED corresponds to the percentage of drug which is drawn from a dosage form and which exits the mouthpiece of an inhaler device.

"Fine particle dose" or "FPD" is defined as the mass percent of powder particles having an aerodynamic diameter less than 3.3 μm, typically determined by measurement in an Andersen cascade impactor. This parameter provides an indication of the percent of particles having the greatest potential to reach the deep lung of a patient for systemic uptake of a drug substance.

A "dispersible" or "dispersive" powder is one having an ED value of at least about 30%, more preferably 40-50%, and even more preferably at least about 50-60%.

"Mass median diameter" or "MMD" is a measure of mean particle size, since the powders of the invention are generally polydisperse (*i.e.*, consist of a range of particle sizes). MMD values as reported herein are determined by centrifugal sedimentation, although any number of commonly employed techniques can be used for measuring mean particle size (e.g., electron microscopy, light scattering, laser diffraction).

"Mass median aerodynamic diameter" or "MMAD" is a measure of the aerodynamic size of a dispersed particle. The aerodynamic diameter is used to describe an aerosolized powder in terms of its settling behavior, and is the diameter of a unit density sphere having the same settling velocity, in air, as the particle. The aerodynamic diameter encompasses particle shape, density and physical size of a particle. As used herein, MMAD refers to the midpoint or median of the aerodynamic particle size distribution of an aerosolized powder determined by cascade impaction, unless otherwise indicated.

"Pharmaceutically acceptable salt" includes, but is not limited to, salts prepared with inorganic acids, such as chloride, sulfate, phosphate, diphosphate,

hydrobromide, and nitrate salts, or salts prepared with an organic acid, such as malate, maleate, fumarate, tartrate, succinate, ethylsuccinate, citrate, acetate, lactate, methanesulfonate, benzoate, ascorbate, para-toluenesulfonate, palmoate, salicylate and stearate, as well as estolate, gluceptate and lactobionate salts. Similarly, salts containing pharmaceutically acceptable cations include, but are not limited to, sodium, potassium, calcium, aluminum, lithium, and ammonium (including alkyl substituted ammonium).

"Pharmaceutically acceptable excipient or carrier" refers to an excipient that may optionally be included in the compositions of the invention, and taken into the lungs with no significant adverse toxicological effects to the subject, and particularly to the lungs of the subject.

"Pharmacologically effective amount" or "physiologically effective amount of a bioactive agent" is the amount of an active agent present in an aerosolizable composition as described herein that is needed to provide a desired level of active agent in the bloodstream or at the site of action (e.g., the lungs) of a subject to be treated to give an anticipated physiological response when such composition is administered pulmonarily. The precise amount will depend upon numerous factors, e.g., the active agent, the activity of the composition, the delivery device employed, the physical characteristics of the composition, intended patient use (i.e., the number of doses administered per day), patient considerations, and the like, and can readily be determined by one skilled in the art, based upon the information provided herein.

"Polymer" refers to a high molecular weight polymeric compound or macromolecule built by the repitition of small, simple chemical units. A polymer may be a biological polymer, i.e., is naturally occurring (e.g., proteins, carbohydrates, nucleic acids) or a non-biological, synthetically-produced polymer (e.g., polyethylene glycols, polyvinylpyrrolidones, Ficolls, and the like), as well known in the art.

II. The Composition

The present invention is based upon the Applicants' discovery of a class of compounds, dipeptides and tripeptides containing two or more leucines, which when incorporated into formulations for administration to the lung, impart superior aerosol

properties to the resulting formulations. Moreover, the Applicants have discovered, surprisingly that, these di- and tripeptides are effective to significantly enhance the dispersibility of the resulting formulations, irrespective of the type of active agent present in the formulation. Thus, these di- and tripeptides can be employed in a wide variety of formulations, to increase the aerosol performance of the resulting compositions, and in some cases, to provide aerosolizable formulations in situations where an aerosolizable formulation was previously unknown or unattainable. The present invention, although directed in certain respects to dry powder formulations, is meant to encompass liquid formulations as well. The components of the formulations of the invention will now be described.

A. The Active Agent

An active agent for incorporation in the compositions described herein may be an inorganic or an organic compound, including, without limitation, drugs which act on: the peripheral nerves, adrenergic receptors, cholinergic receptors, the skeletal muscles, the cardiovascular system, smooth muscles, the blood circulatory system, synoptic sites, neuroeffector junctional sites, endocrine and hormone systems, the immunological system, the reproductive system, the skeletal system, autacoid systems, the alimentary and excretory systems, the histamine system, and the central nervous system. Suitable agents may be selected from, for example, hypnotics and sedatives, psychic energizers, tranquilizers, respiratory drugs, anticonvulsants, muscle relaxants, antiparkinson agents (dopamine antagnonists), analgesics, anti-inflammatories, antianxiety drugs (anxiolytics), appetite suppressants, antimigraine agents, muscle contractants, anti-infectives (antibiotics, antivirals, antifungals, vaccines) antiarthritics, antimalarials, antiemetics, anepileptics, bronchodilators, cytokines, growth factors, anti-cancer agents, antithrombotic agents, antihypertensives, cardiovascular drugs, antiarrhythmics, antioxicants, anti-asthma agents, hormonal agents including contraceptives, sympathomimetics, diuretics, lipid regulating agents, antiandrogenic agents, antiparasitics, anticoagulants, neoplastics, antineoplastics, hypoglycemics, nutritional agents and supplements, growth supplements, antienteritis agents, vaccines, antibodies, diagnostic agents, and

contrasting agents. The active agent, when administered by inhalation, may act locally or systemically.

The active agent may fall into one of a number of structural classes, including but not limited to small molecules, peptides, polypeptides, proteins, polysaccharides, steroids, proteins capable of eliciting physiological effects, nucleotides, oligonucleotides, polynucleotides, fats, electrolytes, and the like.

Examples of active agents suitable for use in this invention include but are not limited to calcitonin, erythropoietin (EPO), Factor VIII, Factor IX, ceredase, cerezyme, cyclosporin, granulocyte colony stimulating factor (GCSF), thrombopoietin (TPO), alpha-1 proteinase inhibitor, elcatonin, granulocyte macrophage colony stimulating factor (GMCSF), growth hormone, human growth hormone (HGH), growth hormone releasing hormone (GHRH), heparin, low molecular weight heparin (LMWH), interferon alpha, interferon beta, interferon gamma, interleukin-1 receptor, interleukin-2, interleukin-1 receptor antagonist, interleukin-3, interleukin-4, interleukin-6, luteinizing hormone releasing hormone (LHRH), factor IX insulin, pro-insulin, insulin analogues (e.g., mono-acylated insulin as described in U.S. Patent No. 5,922,675), amylin, C-peptide, somatostatin, somatostatin analogs including octreotide, vasopressin, follicle stimulating hormone (FSH), insulin-like growth factor (IGF), insulintropin, macrophage colony stimulating factor (M-CSF), nerve growth factor (NGF), tissue growth factors, keratinocyte growth factor (KGF), glial growth factor (GGF), tumor necrosis factor (TNF), endothelial growth factors, parathyroid hormone (PTH), glucagon-like peptide thymosin alpha 1, IIb/IIIa inhibitor, alpha-1 antitrypsin, phosphodiesterase (PDE) compounds, VLA-4 inhibitors, bisphosponates, respiratory syncytial virus antibody, cystic fibrosis transmembrane regulator (CFTR) gene, deoxyreibonuclease (Dnase), bactericidal/permeability increasing protein (BPI), anti-CMV antibody, 13-cis retinoic acid, macrolides such as erythromycin, oleandomycin, troleandomycin, roxithromycin, clarithromycin, davercin, azithromycin, flurithromycin, dirithromycin, josamycin, spiromycin, midecamycin, leucomycin, miocamycin, rokitamycin, andazithromycin, and swinolide A; fluoroquinolones such as ciprofloxacin, ofloxacin, levofloxacin, trovafloxacin, alatrofloxacin, moxifloxicin, norfloxacin, enoxacin, grepafloxacin, gatifloxacin, lomefloxacin, sparfloxacin, temafloxacin,

pefloxacin, amifloxacin, fleroxacin, tosufloxacin, prulifloxacin, irloxacin, pazufloxacin, clinafloxacin, and sitafloxacin, aminoglycosides such as gentamicin, netilmicin, paramecin, tobramycin, amikacin, kanamycin, neomycin, and streptomycin, vancomycin, teicoplanin, rampolanin, mideplanin, colistin, daptomycin, gramicidin, colistimethate, polymixins such as polymixin B, capreomycin, bacitracin, penems; penicillins including penicllinase-sensitive agents like penicillin G, penicillin V, penicllinase-resistant agents like methicillin, oxacillin, cloxacillin, dicloxacillin, floxacillin, nafcillin; gram negative microorganism active agents like ampicillin, amoxicillin, and hetacillin, cillin, and galampicillin; antipseudomonal penicillins like carbenicillin, ticarcillin, azlocillin, mezlocillin, and piperacillin; cephalosporins like cefpodoxime, cefprozil, ceftbuten, ceftizoxime, ceftriaxone, cephalothin, cephapirin, cephalexin, cephradrine, cefoxitin, cefamandole, cefazolin, cephaloridine, cefaclor, cefadroxil, cephaloglycin, cefuroxime, ceforanide, cefotaxime, cefatrizine, cephacetrile, cefepime, cefixime, cefonicid, cefoperazone, cefotetan, cefmetazole, ceftazidime, loracarbef, and moxalactam, monobactams like aztreonam; and carbapenems such as imipenem, meropenem. pentamidine isethiouate, albuterol sulfate, lidocaine, metaproterenol sulfate, beclomethasone diprepionate, triamcinolone acetamide, budesonide acetonide, fluticasone, ipratropium bromide, flunisolide, cromolyn sodium, ergotamine tartrate and where applicable, analogues, agonists, antagonists, inhibitors, and pharmaceutically acceptable salt forms of the above. In reference to peptides and proteins, the invention is intended to encompass synthetic, native, glycosylated, unglycosylated, pegylated forms, and biologically active fragments and analogs thereof.

Active agents for use in the invention further include nucleic acids, as bare nucleic acid molecules, vectors, associated viral particles, plasmid DNA or RNA or other nucleic acid constructions of a type suitable for transfection or transformation of cells, i.e., suitable for gene therapy including antisense. Further, an active agent may comprise live attenuated or killed viruses suitable for use as vaccines. Other useful drugs include those listed within the Physician's Desk Reference (most recent edition).

The amount of active agent in the formulation will be that amount necessary to deliver a therapeutically effective amount of the active agent per unit dose to

achieve the desired result. In practice, this will vary widely depending upon the particular agent, its activity, the severity of the condition to be treated, the patient population, dosing requirements, and the desired therapeutic effect. The composition will generally contain anywhere from about 1% by weight to about 99% by weight active agent, typically from about 2% to about 95% by weight active agent, and more typically from about 5% to 85% by weight active agent, and will also depend upon the relative amounts of additives contained in the composition. The compositions of the invention are particularly useful for active agents that are delivered in doses of from 0.001 mg/day to 100 mg/day, preferably in doses from 0.01 mg/day to 75 mg/day, and more preferably in doses from 0.10 mg/day to 50 mg/day.

It is to be understood that more than one active agent may be incorporated into the formulations described herein and that the use of the term "agent" in no way excludes the use of two or more such agents.

B. Dispersibility-Enhancing Peptides

Compositions of the invention will include one or more di- or tripeptides containing two or more leucine residues. As discussed above, the invention is based upon the Applicants' discovery that di-leucyl-containing dipeptides (e.g., dileucine) and tripeptides are superior in their ability to increase the dispersibility of powdered compositions, and, as demonstrated in the Examples, are unexpectedly better than leucine in improving aerosol performance.

Di-leucyl containing tripeptides for use in the invention are tripeptides having the formula, X-Y-Z, where at least X and Y or X and Z are leucyl residues (i.e., the leucyl residues can be adjacent to each other (at the 1 and 2 positions), or can form the ends of the trimer (occupying positions 1 and 3). The remaining amino acid contained in the trimer can be any amino acid as defined in section I above. Suitable are amino acids such as glycine (gly), alanine (ala), valine (val), leucine (leu), isoleucine (ile), methionine (met), proline (pro), phenylalanine (phe), trytophan (trp), serine (ser), threonine (thr), cysteine (cys), tyrosine (tyr), asparagine (asp), glutamic acid (glu), lysine (lys), arginine (arg), histidine (his), norleucine (nor), and modified forms thereof. Preferably, for di-leucyl containing trimers, the third amino acid

component of the trimer is one of the following: leucine (leu), valine (val), isoleucine (isoleu), tryptophan (try) alanine (ala), methionine (met), phenylalanine (phe), tyrosine (tyr), histidine (his), and proline (pro). Exemplary trimers for use in the invention include but are not limited to the following: leu-leu-gly, leu-leu-ala, leu-leu-val, leu-leu-leu, leu-leu-ile, leu-leu-met, leu-leu-pro, leu-leu-phe, leu-leu-trp, leu-leu-ser, leu-leu-thr, leu-leu-cys, leu-leu-tyr, leu-leu-asp, leu-leu-glu, leu-leu-lys, leu-leu-arg, leu-leu-his, leu-leu-nor, leu-gly-leu, leu-ala-leu, leu-val-leu, leu-ile-leu, leu-met-leu, leu-pro-leu, leu-phe-leu, leu-trp-leu, leu-ser-leu, leu-thr-leu, leu-cys-leu, leu-try-leu, leu-asp-leu, leu-glu-leu, leu-lys-leu, leu-arg-leu, leu-his-leu, and leu-nor-leu. Particularly preferred peptides are dileucine and trileucine.

Although less preferred due to their limited solubility in water, additional dispersibility enhancing peptides for use in the invention are 4-mers and 5-mers containing two or more leucine residues. The leucine residues may occupy any position within the peptide, and the remaining (i.e., non-leucyl) amino acids positions are occupied by any amino acid as described above, provided that the resulting 4-mer or 5-mer has a solubility in water of at least about 1 mg/ml. Preferably, the non-leucyl amino acids in a 4-mer or 5-mer are hydrophilic amino acids such as lysine, to thereby increase the solubility of the peptide in water.

Also preferred are di- and tripeptides having a glass transition temperature greater than about 40 °C.

Preferred di- and tripeptides for use in the present invention are those peptides that are surface active. As can be seen from the surface tension data in Example 1, dileucine and trileucine are extremely effective, even when present in low concentrations, at significantly depressing the surface tension of water. Moreover, in examining the surface tension results results in Table 5 (extrapolated values), it can be seen that dipeptides and tripeptides containing two or more leucines have a much greater surface activity than dipeptides and tripeptides composed of fewer than two leucyl residues. Due to their highly surface active nature, the di- and tripeptides of the invention, when contained in dry powder compositions, tend to concentrate on the surface of the powder particles, thereby imparting to the resulting particles high dispersivities. This feature of the powders,

i.e., a surface enriched with the di- or tripeptide, is illustrated by the ESCA data provided in Example 9.

Surprisingly, the addition of the representative tripeptide, trileucine, to a calcitonin formulation was effective to nearly double the ED value of the resulting powder (Example 4). This result is surprising because calcitonin itself is a surface active protein. Thus, the incorporation of another surface active material such as trileucine was not expected to significantly improve the dispersivity of the composition. Results in contrast to this expectation indicated that surface activity alone is not sufficient to significantly increase dispersibility, and further demonstrated the unusual and beneficial properties of the leucyl-containing peptides of the invention, particularly in enhancing aerosol performance.

Generally, the compositions of the invention will contain from about 1% to about 99% by weight di- or tripeptide, preferably from about 2% to about 75% by weight di- or tripeptide, and even more preferably from about 5% to about 50% by weight di- or tripeptide. Typically, the optimal amount of di- or tripeptide is determined experimentally, i.e., by preparing compositions containing varying amounts of di- or tripeptide (ranging from low to high), examining the dispersibilities of the resulting compositions as described herein, and further exploring the range at which optimal aerosol performance is attained. Such methods were employed in several of the Examples (Example 3, Example 4, Example 5, Example 6). Generally, for trileucine containing dry powder formulations, an optimal amount of trileucine appears to be around 22-25% by weight.

C. Additional Carriers and Excipients

In addition to the active agent and di- or tripeptide, compositions of the invention may optionally include one or more pharmaceutical excipients which are suitable for pulmonary administration. These excipients, if present, are generally present in the composition in amounts ranging from about 0.01 % to about 95% percent by weight, preferably from about 0.5 to about 80%, and more preferably from about 1 to about 60% by weight. Preferably, such excipients will, in part, serve to further improve the features of the active agent composition, e.g., by providing more efficient and reproducible delivery of the active agent, improving the handling characteristics of powders *(e.g.*, flowability and consistency), and/or facilitating

manufacturing and filling of unit dosage forms. In particular, excipient materials can often function to further improve the physical and chemical stability of the active agent, minimize the residual moisture content and hinder moisture uptake, and to enhance particle size, degree of aggregation, particle surface properties (*i.e.*, rugosity), ease of inhalation, and the targeting of particles to the lung. The excipient(s) may also serve simply as bulking agents when it is desired to reduce the concentration of active agent in the formulation.

Pharmaceutical excipients and additives useful in the present composition include but are not limited to amino acids, peptides, proteins, non-biological polymers, biological polymers, carbohydrates (e.g., sugars, derivatized sugars such as alditols, aldonic acids, esterified sugars, and sugar polymers), which may be present singly or in combination. Suitable excipients are those provided in Inhale Therapeutic Systems' International Publication No. WO 96/32096. Also preferred are excipients having glass transition temperatures (Tg), above about 35° C, preferably above about 40 °C, more preferably above 45° C, most preferably above about 55 °C.

Exemplary protein excipients include albumins such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, hemoglobin, and the like. Suitable amino acids (outside of the dileucyl-peptides of the invention), which may also function in a buffering capacity, include alanine, glycine, arginine, betaine, histidine, glutamic acid, aspartic acid, cysteine, lysine, leucine, isoleucine, valine, methionine, phenylalanine, aspartame, tyrosine, tryptophan, and the like. Preferred are amino acids and polypeptides that function as dispersing agents. Amino acids falling into this category include hydrophobic amino acids such as leucine, valine, isoleucine, tryptophan, alanine, methionine, phenylalanine, tyrosine, histidine, and proline. Dispersibility- enhancing peptide excipients include dimers, trimers, tetramers, and pentamers comprising one or more hydrophobic amino acid components such as those described above.

Carbohydrate excipients suitable for use in the invention include, for example, monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose,

cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol), pyranosyl sorbitol, myoinositol and the like.

The compositions may also include a buffer or a pH adjusting agent, typically a salt prepared from an organic acid or base. Representative buffers include organic acid salts of citric acid, ascorbic acid, gluconic acid, carbonic acid, tartaric acid, succinic acid, acetic acid, or phthalic acid, Tris, tromethamine hydrochloride, or phosphate buffers.

The compositions of the invention may also include polymeric excipients/additives, e.g., polyvinylpyrrolidones, derivatized celluloses such as hydroxymethylcellulose, hydroxyethylcellulose, and hydroxypropylmethylcellulose, Ficolls (a polymeric sugar), hydroxyethylstarch, dextrates (e.g., cyclodextrins, such as 2-hydroxypropyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin), polyethylene glycols, and pectin.

The compositions may further include flavoring agents, taste-masking agents, inorganic salts (e.g., sodium chloride), antimicrobial agents (e.g., benzalkonium chloride), sweeteners, antioxidants, antistatic agents, surfactants (e.g., polysorbates such as "TWEEN 20" and "TWEEN 80"), sorbitan esters, lipids (*e.g.*, phospholipids such as lecithin and other phosphatidylcholines, phosphatidylethanolamines), fatty acids and fatty esters, steroids (e.g., cholesterol), and chelating agents (e.g., EDTA, zinc and other such suitable cations). Other pharmaceutical excipients and/or additives suitable for use in the compositions according to the invention are listed in "Remington: The Science & Practice of Pharmacy", 19th ed., Williams & Williams, (1995), and in the "Physician's Desk Reference", 52nd ed., Medical Economics, Montvale, NJ (1998).

III. Formulation Types

The compositions described herein may be in powdered form or may be flowable liquids. Liquid formulations are preferably solutions in which the active drug is dissolved in a solvent (e.g., water, ethanol, ethanol-water, saline) and less

preferably are colloidal suspensions. The liquid formulation may also be a solution or suspension of the active agent in a low boiling point propellant.

Liquid formulations containing the disclosed dileucyl-containing peptides are also highly dispersible, possessing high ED values.

IV. Preparing Dry Powders

Dry powder formulations are preferably prepared by spray drying. Spray drying of the formulations is carried out, for example, as described generally in the "Spray Drying Handbook", 5th ed., K. Masters, John Wiley & Sons, Inc., NY, NY (1991), and in Platz, R., *et al.*, International Patent Publication No. WO 97/41833 (1997), the contents of which are incorporated herein by reference.

Active agents having a solubility in water of at least about 0.10 mg/ml (e.g., peptides, proteins, nucleotides and the like) can be sprayed dried from an aqueous solution. Utilizing this approach, the active agent is first dissolved in water, optionally containing a physiologically acceptable buffer. The pH range of active agent-containing solutions is generally between about 4 and 11, with nearer neutral pHs being preferred, since such pHs may aid in maintaining the physiological compatibility of the powder after dissolution of powder within the lung. The aqueous formulation may optionally contain additional water-miscible solvents, such as acetone, alcohols and the like. Representative alcohols are lower alcohols such as methanol, ethanol, propanol, isopropanol, and the like. The pre-spray dried solutions will generally contain solids dissolved at a concentration from 0.01% (weight/volume) to about 20% (weight/volume), usually from 0.1% to 3% (weight/volume).

The solutions are then spray dried in a conventional spray drier, such as those available from commercial suppliers such as Niro A/S (Denmark), Buchi (Switzerland) and the like, resulting in a dispersible, dry powder. Optimal conditions for spray drying the solutions will vary depending upon the formulation components, and are generally determined experimentally. The gas used to spray dry the material is typically air, although inert gases such as nitrogen or argon are also suitable. Moreover, the temperature of both the inlet and outlet of the gas used to dry the sprayed material is such that it does not cause decomposition of the active agent in

the sprayed material. Such temperatures are typically determined experimentally, although generally, the inlet temperature will range from about 50° C to about 200° C while the outlet temperature will range from about 30° C to about 150° C.

Variations of the above are utilized for spray-drying formulations where the active agent is a hydrophobic drug. One such process is described in Gordon, M.S., Lord, J.D., U.S. Patent No. 5,985,248, assigned to Inhale Therapeutics Systems. In this method, a hydrophobic drug is dissolved in an organic solvent or co-solvent system, and the hydrophilic components (e.g., the leucyl-containing peptides and optional other excipients) are at least partially dissolved in the same organic solvent or co-solvent system. The resulting solution is then spray-dried to form particles. Typically, the solubility of the active agent and the hydrophilic component will govern the selection of the organic solvent system. The organic solvent is selected to provide a solubility for the hydrophilic component of at least 1 mg/ml, and preferably at least 5 mg/ml, and a solubility for the hydrophobic drug of at least 0.01 mg/ml, preferably at least 0.05 mg/ml.

Alternatively, the composition may be prepared by spray-drying a suspension, as described in Gordon, M.S., U.S. Patent No. 5,976,574, assigned to Inhale Therapeutic Systems. In this method, the hydrophobic drug is dissolved in an organic solvent, e.g., methanol, ethanol, isopropanol, acetone, heptane, hexane chloroform, ether, followed by suspension of the hydrophilic excipient in the organic solvent to form a suspension. The suspension is then spray-dried to form particles. Preferred solvents, for both of the above spray-drying methods include alcohols, ethers, ketones, hydrocarbons, polar aprotic solvents, and mixtures thereof.

The dry powders of the invention may also be prepared by combining aqueous solutions or suspensions of the formulation components and spray-drying them simultaneously in a spray-dryer, as described in Gordon, M., U.S. Patent No. 6,001,336, assigned to Inhale Therapeutic Systems. Alternatively, the dry powders may be prepared by preparing an aqueous solution of a hydrophilic excipient or additive, preparing an organic solution of a hydrophobic drug, and spray drying the aqueous solution and the organic solution simultaneously through a nozzle, e.g., a coaxial nozzle, to form a dry powder, as described in Gordon, M., et al, International Publication Number WO 98/29096.

Alternatively, powders may be prepared by lyophilization, vacuum drying, spray freeze drying, super critical fluid processing, air drying, or other forms of evaporative drying. In some instances, it may be desirable to provide the dry powder formulation in a form that possesses improved handling/processing characteristics, e.g., reduced static, better flowability, low caking, and the like, by preparing compositions composed of fine particle aggregates, that is, aggregates or agglomerates of the above-described dry powder particles, where the aggregates are readily broken back down to the fine powder components for pulmonary delivery, as described, e.g., in Johnson, K., *et al.*, U.S. Patent No. 5,654,007, 1997, incorporated herein by reference.

In another approach, dry powders may be prepared by agglomerating the powder components, sieving the materials to obtain agglomerates, spheronizing to provide a more spherical agglomerate, and sizing to obtain a uniformly-sized product, as described, e.g., and in Ahlneck, C., *et al.*, International PCT Publication No. WO 95/09616, 1995, incorporated herein by reference.

Dry powders may also be prepared by blending, grinding, sieving or jet milling formulation components in dry powder form.

Once formed, the dry powder compositions are preferably maintained under dry (*i.e.*, relatively low humidity) conditions during manufacture, processing, and storage. Irrespective of the drying process employed, the process will preferably result in respirable, highly dispersible particles comprising an active agent and a dileucyl-containing dimer or trimer.

V. Features of Dry Powder Formulations

Powders of the invention are further characterized by several features, most notably, (i) consistently high dispersivities, which are maintained, even upon storage (Example 8), (ii) small aerodynamic particles sizes (MMADs), (iii) improved fine particle dose values, i.e., powders having a higher percentage of particles sized less than 3.3 microns MMAD, all of which contribute to the improved ability of the powder to penetrate to the tissues of the lower respiratory tract (*i.e.*, the alveoli) for either localized or systemic treatment. These physical characteristics of the di-leucyl

peptide-containing dry powders, to be described more fully below, are important in maximizing the efficiency of aerosolized delivery of such powders to the deep lung.

Dry powders of the invention are composed of aerosolizable particles effective to penetrate into the lungs. The particles of the invention have a mass median diameter (MMD) of less than about 20 μm, preferably less than about 10 μm, more preferably less than about 7.5 μm, and most preferably less than about 4 μm, and usually are in the range of 0.1 μm to 5 μm in diameter. Preferred powders are composed of particles having an MMD from about 0.2 to 4.0 μm. In some cases, the powder will also contain non-respirable carrier particles such as lactose, where the non-respirable particles are typically greater than about 40 microns in size.

The powders of the invention are further characterized by an aerosol particle size distribution less than about 10 μm mass median aerodynamic diameter (MMAD), and preferably less than 4.0 μm. The mass median aerodynamic diameters of the powders will characteristically range from about 0.1 - 10 μm, preferably from about 0.2 - 5.0 μm MMAD, more preferably from about 1.0 - 4.0 μm MMAD, and even more preferably from about 1.5 to 3.5 μm. Illustrative MMAD values for exemplary di-leucyl-peptide-containing powder compositions are provided in Examples 2, 3, 4, 5, and 6. Several of these examples demonstrate an improvement in aerosol particle size distribution achieved upon incorporation of a di-leucyl di- or tripeptide into the formulation.

The powders of the invention may further be characterized by their densities. The powder will generally possess a bulk density from about 0.1 to 10 g/cubic centimeter, preferably from about 0.1-2 g/cubic centimeter, and more preferably from about 0.15-1.5 g/cubic centimeter.

The powders will generally have a moisture content below about 20% by weight, usually below about 10% by weight, and preferably below about 6% by weight. Such low moisture-containing solids tend to exhibit a greater stability upon packaging and storage.

One of the most striking features of the compositions of the invention is their dispersibility, as indicated by the ED value. The presence of the di-leucyl peptide in the formulations is effective to provide formulations having significantly improved

dispersibilities. Generally, the emitted dose (ED) of these powders is greater than 30%, and usually greater than 40%. More preferably, the ED of the powders of the invention is greater than 50%, and is often greater than 55%. In fact, in looking at the Examples, di-leucyl-peptide containing powders typically possess optimized ED values as high as 80% or above. Moreover, the Examples further illustrate that the incorporation of a di-leucyl di- or tripeptide into a variety of active agent formulations was effective, in all cases, to increase the ED value of the resultant compositions, and in some instances, as much as doubling its value. Moreover, this effect was observed for both protein and small molecule active agent powders.

An additional measure for characterizing the overall aerosol performance of a dry powder is the fine particle dose (FPD), which describes the percentage of powder having an aerodynamic diameter less than 3.3 microns. The powders of the invention are particularly well suited for pulmonary delivery, and possess FPF values ranging from about 35%-85%. Such powders contain at least about 35 percent of aerosol particle sizes below 3.3 μm to about 0.5 μm and are thus extremely effective when delivered in aerosolized form, in reaching the regions of the lung, including the alveoli.

The compositions described herein also possess good stability with respect to both chemical stability and physical stability, i.e., aerosol performance, over time (Example 8). Generally, with respect to chemical stability, the active agent contained in the formulation will degrade by no more than about 10% over a time course of three months, preferably by no more than about 7%, and more preferably by no more than 5%, upon storage of the composition under ambient conditions. As illustrated by the exemplary PTH formulation in Example 8, storage under accelerated stability conditions (40 °C, ambient humidity) for over a period of 3 months (12 weeks) resulted in the degradation of only 2.3% protein (from an initial value of 97.1% purity to 94.8% purity). Since accelerated temperatures result in an increase in reaction rate, one can conclude that storage of the same composition under ambient conditions would result in a degradation rate less than 2.3%, further pointing to the chemical stability of the present compositions.

With respect to aerosol performance, compositions of the invention are generally characterized by a drop in emitted dose of no more than about 20%,

preferably no more than about 15%, and more preferably by no more than about 10%, when stored under ambient conditions for a period of three months. In looking at the results in Example 8, an exemplary PTH-trileucine formulation exhibited essentially no change, and in particular, no diminishment, in aerosol properties (MMAD, FPD, ED) upon storage under accelerated stability conditions (40 °C, ambient humidity).

Another preferred feature of particulate compositions of the invention is an enrichment of the di-leucyl di- or tripeptide on the surface of the particles, as indicated by the results in Example 9.

The improvement in aerosol properties discovered for di-leucyl di- and tripeptide-containing composition (i.e., greatly enhanced dispersibilities, reduced fine particle dose values, smaller aerodynamic diameters), can result in several related advantages, such as: (i) reducing costly drug loses to the inhalation device, since more powder is aerosolized and is therefore available for inhalation by a subject; (ii) reducing the amount of dry powder required per unit dose, due to the high efficiency of aerosolization of powder, (iii) reducing the number of inhalations per day by increasing the amount of aerosolized drug reaching the lungs of a subject.

VI. Administration of the Composition

The formulations described herein may be delivered using any suitable dry powder inhaler (DPI), *i.e.*, an inhaler device that utilizes the patient's inhaled breath as a vehicle to transport the dry powder drug to the lungs. Preferred are Inhale Therapeutic Systems' dry powder inhalation devices as described in Patton, J.S., *et al.*, U.S. Patent No. 5,458,135 (1995) Smith, A.E., *et al.*, U.S. Patent No. 5,740,794 (1998); and in Smith, A.E., *et. al.*, U.S. Patent No. 5,785,049 (1998), herein incorporated by reference.

When administered using a device of this type, the powder is contained in a receptacle having a puncturable lid or other access surface, preferably a blister package or cartridge, where the receptable may contain a single dosage unit or multiple dosage units. Convenient methods for filling large numbers of cavities (i.e., unit dose packages) with metered doses of dry powder medicament are described, e.g., in Parks, D.J., *et al.*, WO 97/41031 (1997) incorporated herein by reference.

Also suitable for delivering the powders described herein are dry powder inhalers of the type described, for example, in Cocozza, S., *et al.*, U.S. Patent No. 3,906,950 (1974), and in Cocozza, S., *et al.*, U.S. Patent No. 4,013,075 (1997), incorporated herein by reference, wherein a premeasured dose of dry powder for delivery to a subject is contained within a hard gelatin capsule.

Other dry powder dispersion devices for pulmonarily administering dry powders include those described, for example, in Newell, R.E., *et al,* European Patent No. EP 129985 (1988); in Hodson, P.D., *et al.*, European Patent No. EP 472598 (1996); in Cocozza, S., *et al.*, European Patent No. EP 467172 (1994), and in Lloyd, L.J. *et al.*, U.S. Patent No. 5,522,385 (1996), incorporated herein by reference. Also suitable for delivering the dry powders of the invention are inhalation devices such as the Astra-Draco "TURBUHALER". This type of device is described in detail in Virtanen, R., U.S. Patent No. 4,668,281 (1987); in Wetterlin, K., *et al* U.S. Patent No. 4,667,668 (1987); and in Wetterlin, K., *et al.* U.S. Patent No. 4,805,811 (1989), all of which are incorporated herein by reference. Other suitable devices include dry powder inhalers such as the Rotahaler® (Glaxo), Discus® (Glaxo), Spiros™ inhaler (Dura Pharmaceuticals), and the Spinhaler® (Fisons). Also suitable are devices which employ the use of a piston to provide air for either entraining powdered medicament, lifting medicament from a carrier screen by passing air through the screen, or mixing air with powder medicament in a mixing chamber with subsequent introduction of the powder to the patient through the mouthpiece of the device, such as described in Mulhauser, P., *et al,* U.S. Patent No. 5,388,572 (1997), incorporated herein by reference.

Dry powders may also be delivered using a pressurized, metered dose inhaler (MDI), e.g., the Ventolin® metered dose inhaler, containing a solution or suspension of drug in a pharmaceutically inert liquid propellant, e.g., a chlorofluorocarbon or fluorocarbon, as described in Laube, *et al.*, U.S. Patent No. 5,320,094 (1994), and in Rubsamen, R.M., *et al*, U.S. Patent No. 5,672,581 (1994), both incorporated herein by reference. Alternatively, the powders described herein may be dissolved or suspended in a solvent, e.g., water, ethanol, or saline, and administered by nebulization. Nebulizers for delivering an aerosolized solution

include the AERx™ (Aradigm), the Ultravent® (Mallinkrodt), and the Acorn II® (Marquest Medical Products).

Prior to use, dry powders are generally stored under ambient conditions, and preferably are stored at temperatures at or below about 25°C, and relative humidities (RH) ranging from about 30 to 60%. More preferred relative humidity conditions, e.g., less than about 30%, may be achieved by the incorporation of a dessicating agent in the secondary packaging of the dosage form.

VII. Utility

The compositions of the invention are useful, when administered pulmonarily in a therapeutically effective amount to a mammalian subject, for treating or preventing any condition responsive to the administration of an active agent as described in section II.A above.

The following examples are illustrative of the present invention, and are not to be construed as limiting the scope of the invention. Variations and equivalents of this example will be apparent to those of skill in the art in light of the present disclosure, the drawings and the claims herein.

All articles, books, patents and other publications referenced herein are hereby incorporated by reference in their entirety.

Examples

Materials and Methods

A. Materials.

Ciprofloxacin Hydrochloride (Neuland Laboratories, India).

Gentamicin Sulfate (H&A (Canada) Industrial)

Netilmicin Sulfate (Scientific Instruments And Technology)

L-Leucine (Aldrich, St. Louis, MO)

Hydrochloric Acid (J. T. Baker, Phillipsburg, N.J.)

Sodium Hydroxide 0.1N Volumetric Solution (J. T. Baker, Phillipsburg, N.J.)

Ethanol, 200 proof (USP/NF, Spectrum Chemical Mfg. Corp., New Brunswick, N.J.)

Methanol (HPLC grade, EM Industries, Gibbstown, N.J.)

S. calcitonin (Bachem California Inc, USA Torrance, CA).

Trileucine (Bachem California Inc, USA Torrance, CA).

Other amino acids used in surface tension experiments were obtained from Sigma St. Louis, MO.

B. Methods.

Particle size measurements (Horiba)

Mass median diameters (MMD) of the powders were measured using a Horiba CAPA-700 particle size analyzer (Horiba Instruments inc., Irvine, CA). Measurements were based upon centrifugal sedimentation of dispersed particles in suspending medium. Mass median diameter, which is based on the particle's Stokes' diameter, was calculated using the particle density and the density and viscosity of the suspending medium.

The density of the powder was set as 1.5 g/cm^3 for all powders. (This nominal value was used for all powders analyzed and is within a range that is typical for spray dried powders). Particle size measurements were conducted with about 5 - 10 mg powder suspended in 5 ml Sedisperse A-11 (Micromeritics, Norcross, GA) and dispersed by sonication for 10 minutes. The range over which particle size data was gathered was set to 0.4 to 10.0 μm.

Aerodynamic Particle Size Measurements

Andersen Cascade Impactor. An Andersen cascade impactor (a sieve-like apparatus with a series of stages that capture particles on plates by inertial impaction according to their size) was used to determine the MMAD and particle size distribution of aerosolized powder formulations in an air stream. The plates were weighed before and after testing and the mass of powder deposited on the plate of each stage was determined. Unless otherwise indicated, studies were undertaken using a traditional Andersen cascade impactor having eight stages (from top to bottom stages 0 to 7) with cut-off sizes ranging from 9.0 to 0.4 μm, and a final filter stage that traps particles < 0.4 μm when operated at a flow rate of 28.3 L/min. The

device test set-up was similar to the ED test, except that the cascade impactor and a USP (United States Pharmacopia) throat (USP 23, chapter <601>) were attached to the device mouthpiece rather than to a filter. Multiple dispersions were typically conducted for each cascade impaction run to achieve gravimetrically accurate data.

Andersen Short Stack (SS) Method. In the SS method, the order in which the stages were placed were altered from the conventional Andersen cascade impactor set-up as described above. From the top, stage 0 was utilized for inlet cone attachment to connect the throat. Stage 3 was positioned next, beneath stage 0, followed by the filter stage (stage F). The powder-containing airstream passes only through stages 0 and 3; air (but not powder) flows through the other stages, which are placed under stage F to hold the remainder of the assembly in place. A pre-weighed filter was placed on stage F and captured particles < 3.3 μm. A second filter was placed on an inverted plate under stage 3, and captured particles > 3.3 μm. For the studies described herein, one BP (blister pack) containing 2 mg of powder composition was dispersed in an aerosol delivery device and a vacuum was pulled at 28.3 L/min as per USP methodology. This process was then repeated two times for a target mass of 6 mg per run. The filters were then removed and weighed to determine the amount of powder deposited.

Example 1
Surface Activity of Di- and Tripeptides

The surface tension of several representative dipeptides, tripeptides, and proteins was measured at 25 °C and 45 °C to provide an indication of their relative surface activities. Surface tension measurements were carried out using a Kruss Processor Tensiometer-K12 with the Wilhelmy-method (Plate method).

Solutions were prepared by dissolving either 0.05%, 0.2%, or 0.6% peptide/protein (by weight) along with an appropriate amount of raffinose by weight to provide final solutions having a 1.0% by weight solids content. Surface tension measurements at 25 °C and 45 °C were then obtained for the test solutions at three different time points (49 seconds, 100 seconds and 194 seconds). The results are shown in Tables 1-5 below.

Highly surface active peptides and proteins are those that are effective to lower the surface tension of water from its control value(s). As can be seen in Tables 1-4, raffinose (which was added to each of the solutions to bring the overall solids content to 1.0%) is non-surface active, and thus does not impact the surface tension results obtained for each of the peptides/proteins.

In looking at the results below, it can be seen that highly surface active peptides include the peptides, dileucine and trileucine. These peptides were as effective as the highly surface active protein, salmon calcitonin, at significantly lowering the surface tension of water. Trileucine was effective at lowering the surface tension of water to a greater extent at higher concentrations (see, for example, data for 0.05%, 0.2% and 0.6% by weight tri-leucine). In comparison to trileucine and dileucine, the dimer of isoleucine and the dimer and trimer of valine were not particularly effective at lowering the surface tension of water.

This method can be used to identify additional surface active di- and tri-peptides suitable for use in the dry powders of the invention.

Table 1. Surface Tension Measurements

SAMPLE	ST, mN/m	time,s	ST, mN/m	time, s	St, mN/m	time, s
water blank-1	72.6	49	72.6	100	72.6	194
water blank-2	72.5	49	72.5	100	72.4	194
water blank-3	72.5	49	72.4	100	72.4	194
1% raffinose-1	72	49	72	100	72	194
1% raffinose-2	72	49	72	100	72	194
1% raffinose-3	72	49	72	100	72	194
0.2% tri-alanine-1	72.4	49	72.4	100	72.3	194
0.2% tri-alanine-2	72.2	49	72.2	100	72.2	194
0.2% tri-alanine-3	72.3	49	72.2	100	72.2	194
0.2% tri-glutamate-1	72.1	49	72.1	100	72.1	194
0.2% tri-glutamate-2	72.4	49	72.4	100	72.4	194
0.2% tri-glutamate-3	72.4	49	72.4	100	72.3	194
0.2% di-alanine-1	72	49	72	100	71.9	194
0.2% di-alanine-2	72	49	71.9	100	71.9	194
0.2% di-alanine-3	72.1	49	72.1	100	72.1	194
0.2% di-leucine-1	58.4	49	58.1	100	57.9	194
0.2% di-leucine-2	58.7	49	58.3	100	58.2	194
0.2% di-leucine-3	60.1	49	59.8	100	59.7	194
0.2% tri-leucine-1	51	49	50.9	100	50.9	194
0.2% tri-leucine-2	51	49	50.8	100	50.7	194
0.2% tri-leucine-3	51	49	50.8	100	50.7	194
0.2% sal. Calcitonin-1	48.7	49	48.6	100	48.5	194
0.2% sal.calcitonin-2	48.4	49	48.4	100	48.4	194
0.2% sal.calcitonin-3	48.4	49	48.4	100	48.4	194

Measurements conducted at 25 °C. The 0.2% (wt/vol) solutions additionally contain raffinose to form solutions having a total solids content of 1% (wt/vol).

Table 2. Surface Tension Measurements

SAMPLE	ST, mN/m	time, s	ST, mN/m	time, s	St, mN/m	time, s
water blank-1	72	49	71.8	100	71.7	194
water blank-2	72.2	49	72.2	100	72.2	194
water blank-3	71.5	49	71.6	100	71.6	194
0.2% di-isoleucine-1	67.6	49	67.2	100	67	194
0.2% di-isoleucine-2	68	49	67.8	100	67.6	194
0.2% di-isoleucine-3	67.7	49	71.6	100	71.6	194
0.2% di-valine-1	71.7	49	71.6	100	71.6	194
0.2% di-valine-2	71.6	49	71.6	100	71.6	194
0.2% di-valine-3	71.7	49	71.6	100	71.6	194
0.2% tri-valine-1	68.8	49	68.8	100	68.8	194
0.2% tri-valine-2	68.8	49	68.7	100	68.7	194
0.2% tri-valine-3	68.7	49	68.7	100	68.7	194

Surface tension measurements conducted at 25 °C.

Solutions contained 0.20% (wt/vol) of one of: di-isoleucine, di-valine, or tri-valine and 0.80% (wt/vol) raffinose.

Table 3. Surface Tension Measurements

SAMPLE	ST, mN/m	time, s	ST, mN/m	time, s	St, mN/m	time, s
1% raffinose (pH4)-1	71.4	49	71.4	100	71.4	194
1% raffinose (PH4)-2	71.1	49	71.1	100	71.1	194
1% raffinose (pH4)-3	71.1	49	71.1	100	71.1	194
1% raffinose(pH7)-1	71.1	49	71.1	100	71.1	194
1% raffinose (pH7)-2	71.1	49	71.1	100	71.1	194
1% raffinose (pH7)-3	71.1	49	71.1	100	71.1	194
water blank-1	72.1	49	72	100	72	194
water blank-2	72.2	49	72.1	100	72	194
water blank-3	72.2	49	72.1	100	72	194
0.05% leu3(pH4)-1	59.9	49	59.8	100	59.7	194
0.05% leu3(pH4)-2	60.4	49	60.3	100	60.2	194
0.05% leu3(pH4)-3	60.4	49	60.3	100	60.2	194
0.2% leu3(pH4)-1	51.4	49	51.2	100	51.1	194
0.2% leu3(pH4)-2	51.4	49	51.3	100	51.2	194
0.2% leu3(pH4)-3	51.4	49	51.2	100	51.1	194
0.6% leu3(pH4)-1	44.2	49	44.1	100	44	194
0.6% leu3(pH4)-2	44.3	49	44.2	100	44.2	194
0.6% leu3(pH4)-3	44.2	49	44.2	100	44.1	194
0.05% leu3(pH7)-1	60.1	49	59.8	100	59.7	194
0.05% leu3(pH7)-2	60	49	59.8	100	59.7	194
0.05% leu3(pH7)-3	60.2	49	60	100	59.8	194
0.2% leu3(pH7)-1	51	49	50.8	100	50.7	194
0.2% leu3(pH7)-2	50.9	49	50.7	100	50.6	194
0.2% leu3(pH7)-3	50.7	49	50.5	100	50.4	194
0.6% leu3(pH7)-1	43.7	49	43.7	100	43.6	194
0.6% leu3(pH7)-2	43.8	49	43.7	100	43.7	194
0.6% leu3(pH7)-3	43.8	49	43.7	100	43.7	194
water blank-5	71.7	49	71.7	100	71.6	194
water blank-6	72.2	49	72.1	100	72.1	194

Surface tension measurements measured at 25 °C. The trileucine formulations also contain raffinose to provide solutions having a total solids content of 1% (wt/vol).

Table 4. Surface Tension Measurements

SAMPLE	ST, mN/m	time, s	ST, mN/m	time, s	St. mN/m	time, s
water blank-5	69.2	49	69.2	100	69.2	194
1% raffinose(pH4)-1	67.9	49	68	100	68	194
1% raffinose (pH4)-2	68.2	49	68.2	100	68.2	194
1% raffinose (pH4)-3	68	49	68	100	68.1	194
1% raffinose (pH7)-1	68.3	49	68.3	100	68.3	194
1% raffinose (pH7)-2	68.4	49	68.4	100	68.4	194
1% raffinose (pH7)-3	68.4	49	68.4	100	68.4	194
Leu3 formulations contain raffinose to make 1% total solids content						
0.05% leu3(pH4)-1	57.1	49	57	100	57	194
0.05% leu3(pH4)-2	58.1	49	57.9	100	57.8	194
0.05% leu3(pH4)-3	58	49	57.8	100	57.8	194
0.2% leu3(pH4)-1	47.9	49	47.5	100	47.4	194
0.2% leu3(pH4)-2	47.2	49	47.2	100	47.3	194
0.2% leu3(pH4)-3	47.9	49	47.3	100	47.1	194
0.6% leu3(pH4)-1	40.9	49	40.9	100	40.8	194
0.6% leu3(pH4)-2	41.1	49	41	100	40.9	194
0.6% leu3(pH4)-3	41.1	49	41	100	40.8	194
0.05% leu3(Ph7)-1	58.5	49	58.4	100	58.4	194
0.05% leu3(pH7)-2	58.2	49	58.2	100	58.1	194
0.05% leu3(pH7)-3	58.2	49	58.1	100	58.1	194
0.2% leu3(pH7)-1	58.5	49	58.4	100	58.4	194
0.2% leu3(pH7)-2	58.2	49	58.2	100	58.1	194
0.2% leu3(pH7)-3	58.2	49	58.1	100	58.1	194

Surface tension measurements taken at 45 °C. Tri-leucine-containing formulations also contain raffinose to provide a solution having a total solids content of 1%.

Additional surface tension measurements were obtained to determine dimers and trimers for use in the invention (i.e., surface active dimers and trimers).

Table 5. Surface Tension of Representative Dimers and Trimers

		25 °C		45 °C		25 °C	45 °C
		Actual		Actual		Extrapolated Values	Extrapolated Values
SAMPLE	concentration mg/ml	MEAN	SD	MEAN	SD	at 2 mg/ml	at 2 mg/ml
Dimers							
Leu-2	13.60	46.6	0.6	42.7	0.3	60.4	52.4
	4.53	54.9	0.5	48.2	0.1		
	1.51	61.5	0.6	53.2	0.1		
Leu-Val	8.80	59.1	0.2	55.7	0.3	67.2	62.3
	2.93	65	0.2	60.4	0.0		
	0.98	69.2	0.4	64.2	0.2		
Leu-Tyr	6.40	62.2	0.1	59.5	0.3	68.3	67.3
	2.13	68.0	0.1	65.6	0.3		
	0.71	71.5	0	68.0	0.1		
Val-Leu	7.80	68	0	63.5	0.2	69.8	65.3
	2.60	69.5	0.1	65.0	0.2		
	0.87	70	0.5	65.5	0.0		
Val-Ile	10.00	66.1	0	61.9	0.2	70.3	65.8
	3.33	70.1	0.3	65.3	0.1		
	1.11	71.6	0.2	66.3	0.2		
Leu	TBD	56.7	0.3	54.7	0.2		
		66.3	0.2	61.4	0.2		
Trimers		70.8	0.2	64.2	0.0		
Leu-Tyr-Leu	2.90	44.7	0.1	40.8	0.0	47.9	44.7
	0.97	51.6	0.1	49.1	0.1		
	0.32	58.4	0.1	55.4	0.3		
Leu-Phe-Leu	6.10	41.5	0.2	39.3	0.0	48.3	46.2
	2.03	48.3	0	46.2	0.1		
	0.68	54.7	0.1	53.6	0.1		
Leu-3	6.10	42.4	0	38.9	0.2	49.7	46.3
	2.03	49.7	0	46.3	0.3		
	0.68	56.9	0	52.8	0.6		
Leu-Leu-Ala	6.80	39.9	0.5	48.4	0.2	46.6	49.8
	2.27	43.5	0.8	48.2	4.3		
	0.76	60.7	0.4	58.3	0.5		
Ala-Val-Leu	8.70	55.7	0.2	53.8	0.0	65	58.9
	2.90	62.8	0.5	57.7	0.2		
	0.97	67.5	0.5	60.3	0.1		

As can be seen from the above, surface active dimers and trimers are more effective when present at higher concentrations at lowering the surface tension of water. As an example, at a concentration of 1.20 mg/ml, the presence of trileucine was effective to lower the surface tension of water from about 72 mN/m to 42 mN/s, while at a concentration of 0.68 mg/ml, trileucine was effective at lowering the surface tension of water to about 57 mN/m.

To normalize for concentration effects, surface tension values were extrapolated to solutions having a concentration of 2 mg/ml (Table 5, columns 7 and 8). Looking first at the dimers, dileucine was more effective than any of the other dimers examined in reducing the surface tension of water. Looking at data for the trimers, leu-tyr-leu is the most surface active of the trimers. Trimers containing, in addition to two leucyl residues, a hydrophobic amino acid such as tyrosine, phenylalanine, leucine, or alanine, are more surface active than trimers containing fewer than two leucyl residues.

In summary, dimers and trimers containing two or more leucines were effective at significantly lowering the surface tension of water (e.g., leu-try-ala, leu-phe-leu, leu-leu-leu, leu-leu-ala, and the like), and are preferred for use in the compositions of the invention.

Example 2

Aerosol Properties of a Parathyroid Hormone (PTH)-Trileucine Dry Powder

Dry powders containing an illustrative active protein, parathyroid hormone, in combination with either leucine or tri-leucine, were prepared. Also prepared was a dry powder absent either leucine or trileucine, to demonstrate the notable improvement in aerosol properties upon addition of trileucine.

Representative PTH powders were prepared as follows.

A. Solution Formulation Preparation

Aqueous formulation solutions were prepared at a total solids content of 1% (w/v). The pH of each solution was determined, and solutions were then spray-dried. Table 6 lists the compositions of all pre-spray-dried PTH solutions.

B. Powder Processing: Spray Drying

Powders were produced by spray drying aqueous solutions of PTH as described in A. above using a Buchi 190 mini spray dryer (Buchi Labortechnik AG, Meierseggstrasse, Switzerland) equipped with a customized nozzle (Platz, R., *et al.*, Inhale Therapeutic Systems' International Patent Publication No. WO 97/41833, Nov. 13, 1997) and cyclone. High collection efficiencies (yields), usually between about 50-80%, were attained.

Table 6. PTH Dry Powder Compositions

Lot No.	Com-position	Emitted Dose, % mean n=10	RSD, %	MMAD (μm)	FPD
R97190	30% PTH 70% mannitol	62	4	-	-
	30% PTH 70% raffinose	66	9	-	-
R97191	75% PTH 25% mannitol	51	3	-	-
	30% PTH 70% leu	78	-	2.43	0.58
	30% PTH 70% tri-leu	83	-	2.63	0.45

In looking at the results in Table 6 (and in other tables as well), it can be seen that the addition of trileucine is effective to significantly improve the aerosol performance of the resulting powder. The aerosol performance of a PTH dry powder, as indicated by its ED value, was unexpectedly increased from 51-62% to 83% by the addition of tri-leucine to the formulation. These data illustrate a tremendous improvement in emitted dose, achieved simply by addition of the exemplary surface active tripeptide, tri-leucine to the formulation. Surprisingly, even upon correcting on a mole-to-mole basis for the number of leucine amino acids contained in trileucine (3 moles leu per mole of trileucine), trileucine is more effective than leucine, on a per weight basis, at increasing the dispersivity of dry powder compositions for delivery to the lung.

Example 3

Aerosol Properties of Albuterol-Trileucine Dry Powders

Dry powders containing the small molecule, albuterol, were prepared to examine the effects of trileucine on the dispersivity/aerosol properties of dry powders containing a non-proteinaceous active agent.

A. Solution Formulation Preparation

Formulation solutions were prepared at a total solids content of 1% (w/v). For low solids-containing solutions, raffinose was added to bring the total solids content to the above value. Table 7 lists the compositions of all pre-spray dried solutions.

37. Powder Processing: Spray Drying

Powders were produced by spray drying aqueous solutions of albuterol, surface active di- or tri-peptide, and/or other excipient(s) using a Buchi 190 mini spray dryer (Buchi Labortechnik AG, Meierseggstrasse, Switzerland) as described in Example 2 above. Characteristics of the resultant powders are provided in Tables 7 and 8 below.

Table 7. Albuterol Dry Powders

Formulation	Emitted Dose, %	Tg, °C
2% albuterol 98% raffinose	31	102.2
2% albuterol 5% leucine raffinose	31	88.57
2% albuterol 20% leucine raffinose	34	93.1
2% albuterol 60% leucine raffinose	74	96.6
2% albuterol 5% trileucine raffinose	62	85.3
2% albuterol 20% trileucine raffinose	78	95.9
2% albuterol 60% trileucine raffinose	82	88.6

Table 8. Additional Aerosol Properties of Albuterol Dry Powders

Formulation	FPD	MMAD, microns
2% albuterol 60% leucine raffinose	0.56	2.43
2% albuterol 20% tri-leucine raffinose	0.59	2.43

As can be seen from the results provided above, the addition of trileucine increased the emitted dose of albuterol dry powders from about 30% to about 80% - an improvement in dispersivity of nearly three-fold! Thus, the addition of a surface active di- or tri-peptide to an active agent dry powder can, by greatly improving the powder's dispersivity, (i) reduce costly drug loses to the inhalation device, (ii) reduce the number of required inhalations per day by increasing the amount of aerosolized drug reaching the alveoli of a patient, (iii) reduce the amount of dry powder per unit dose, due to the high efficiency of aerosolization of dry powder, and (iv) increase the ease of manufacturing unit dosage forms of powdered drug, due to increased flowability of powder.

Additionally, the addition of 60% by weight leucine was required to achieve the same level of dispersivity achieved by the addition of only 20% by weight tri-leucine. Thus, tri-leucine is much more effective than leucine in improving the aerosol performance of dry powders. Moreover, a maximum in aerosol performance is typically achieved by the addition of only from about 5-25% (wt) trileucine; quantities greater than that typically provide only incremental improvements in dispersivity.

The dispersibility-enhancing effects of tri-leucine, and other surface active di- and tri-peptides, appear to be general, and extend to not only protein powders, but to powdered formulations of a wide variety of active agents (e.g., small molecules, hormones, antibiotics, and the like), as illustrated by the Examples provided herein.

Example 4
Aerosol Properties of Salmon Calcitonin-Trileucine Dry Powders

The effects of trileucine on the aerosol performance of dry powders containing salmon calcitonin, a hormone with a molecular weight of approximately 4500 daltons, were examined.

Although salmon calcitonin is a highly surface active protein, spray-dried powders containing 5% (wt) salmon calcitonin and 95% (wt) raffinose exhibited relatively low emitted dose values (of approximately 50%). In efforts to further explore the broad applicability of adding surface active di- and tri-peptides to powder formulations to increase their dispersivity, tri-leucine was added to salmon calcitonin-containing formulations to examine its impact on the resulting powders. The ability of tri-leucine to improve the dispersibility of salmon calcitonin containing dry powders was compared to the amino acid, leucine.

Powders having the compositions indicated below were prepared as described in Examples 2 and 3 above.

Table 9. S. Calcitonin Dry Powders

Formulation	Emitted Dose	FPD	Tg, °C
5% s. Calcitonin 95% raffinose	48	0.30	89.9
5% s. Calcitonin 5% leucine raffinose	47	0.31	89.3
5% s. Calcitonin 20% leucine raffinose	50	0.28	82.9
5% s. Calcitonin 40% leucine raffinose	48	0.29	82.3
5% s. Calcitonin 60% leucine raffinose	53	0.22	80.5
5% s. Calcitonin 80% leucine raffinose	64	0.29	74.5
5% s. Calcitonin 5% tri-leucine raffinose	58	0.46	89
5% s. Calcitonin 20% tri-leucine raffinose	72	0.50	91.1
5% s. Calcitonin 40% tri-leucine raffinose	76	0.46	83.4
5% s. Calcitonin 60% tri-leucine raffinose	84	0.49	94.3
5% s. Calcitonin 80% tri-leucine raffinose	86	0.49	115.2

Representative mass median aerodynamic diameters were determined for two of the formulations.

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Table 10. Mass Median Aerodynamic Diameters of Calcitonin Powders

Formulation	MMAD
5% s. Calcitonin 20% leucine raffinose	3.39
5% s. Calcitonin 20% tri-leucine raffinose	2.87

From the above data, it can be seen that tri-leucine can be used to improve the aerosol properties of dry powder formulations of a wide range of active agents/medicaments for aerosolized delivery to the lung.

Trileucine provided nearly a 100% improvement in the emitted dose value of a control powder containing salmon calcitonin and raffinose, nearly doubling its ED value from 48% to 86%. Moreover, tri-leucine was more effective in enhancing powder dispersibility than leucine. While a representative formulation containing 80% by weight leucine exhibited an ED value of 64%, formulations containing 60-80% tri-leucine possessed ED values from 84-86%, further indicating the superiority of tri-leucine in significantly enhancing the aerosol performance of dry powders.

Example 5
Aerosol Properties of Antibiotic-Trileucine Dry Powders

The ability of tri-leucine to improve the dispersibility of antibiotic-containing dry powders was explored.

A. Antibiotic Control Powders Absent Trileucine

Ciprofloxacin Powders. Aqueous solutions containing the components presented in Table 9 were prepared at a total solids content of 1% (w/v). The pH of each solution was determined, and solutions were then spray-dried as described in Example 2 to prepare dry powders.

Table 11.

Batch Number	Quantitative Composition Prior to Spray Drying[1]	Moisture Content	MMAD (μm)	Emitted Dose
(1) 1326-16	Ciprofloxacin hydrochloride 1136 mg DI water 113 ml solid product: 100% cipro	1.4%	2.8	42% (RSD=8)
(2) 1326-29	Ciprofloxacin hydrochloride 2047 mg DI water 200 ml Sodium hydroxide QS to pH=12 solid product: 100% cipro	3.2%	4.5	51% (RSD=7)
(3) 1300-MG-7	Ciprofloxacin hydrochloride 1995 mg Methanol 100 ml DI water 100 ml solid product: 100% cipro	1.2%	2.9	33% (RSD=13)

Gentilmicin, Netilmicin Powders.

Dry powder compositions containing gentamicin or netilmicin were prepared by mixing gentamicin sulfate or netilmycin sulfate and excipient(s) (if used) with a liquid medium to form a solution. The pH of the solution was adjusted as appropriate to facilitate solubilization and/or stabilization of the components in the solution. Quantitative formulations are identified in Table 12 below. The solutions were then spray-dried as described in Example 2 above to yield dry powders. For formulations that utilized organic solvents, a modified Buchi 190 Mini Spray Dryer was used that was supplied with nitrogen as the gas source and equipped with an oxygen sensor and other safety equipment to minimize the possibility of explosion.

Table 12. Gentamicin/Netilmicin Dry Powders

Batch Number	Quantitative Composition		Moisture Content	MMAD (μm)	Emitted Dose
1326-31	Gentamicin sulfate DI water Hydrochloric acid	2076 mg 200 ml QS to pH=5	4.1%[1]	3.0	37% (RSD[3]=6)
1326-32	Gentamicin sulfate DI water Sodium hydroxide	2053 mg 200 ml QS to pH=10	1.1%[1]	2.4	40% (RSD=14)
1300-MG-11	Gentamicin sulfate Ethanol DI water	2012 mg 40 ml 160 ml	4.8%[2]	3.0	45% (RSD=10)
1300-MG-9	Netilmicin Sulfate DI water	1626 mg 163 ml	4.2%	3.2	47% (RSD=8)
1300-MG-14	Netilmicin Sulfate Ethanol DI water	1512 mg 30 ml 120 ml	5.1%	2.9	39% (RSD=7)

[1] Determined with Karl-Fischer reagent titrimetric method
[2] Determined with thermogravimetric analysis
[3] Relative Standard Deviation

B. Trileucine-Containing Antibiotic Powders

Aqueous solutions (100 ml total volume) containing antibiotic and tri-leucine at a total solids content of 1% were prepared and the pH of the solutions adjusted to pH 4. The resulting solutions were then spray-dried to produce powders having the relative amounts of antibiotic and tri-leucine indicated in Table 13 below.

Table 13. Antibiotic-Trileucine Dry Powders

Formulation	Yield, %	MMAD, μm	FPD (<3.3 μm)	ED, %
95% Cipro 5% Leu-3	64.2	2.43	0.57	77.7
75% Cipro 25% Leu-3	N.A.	2.65	N.A.	83.0
45% Cipro 55% Leu-3	55.0	2.62	0.48	70.7
95% Gent. 5% Leu-3	61.4	2.15	0.66	75.7
75% Gent. 25% Leu-3	52.0	2.25	0.66	93.9
55% Gent. 45% Leu-3	54.2	2.51	0.51	87.3
95% Netil. 5% Leu-3	62.0	2.08	0.58	82.4
75% Netil. 25% Leu-3	50.0	2.14	0.66	91.3
55% Netil. 45% Leu-3	40.0	2.73	0.49	90.4

As can be seen from the results in Table 13, the addition of tri-leucine was effective to notably enhance the dispersibility of powders prepared from three different antibiotic compounds from two different antibiotic classes, ciprofloxacin (a quinolone), gentamicin and netilmicin (aminoglycosides). The ED values for ciprofloxacin powders increased from values ranging from 33-51% to values ranging from 71-83%. Similar beneficial results were observed for gentamicin powders, whose ED values were improved from 37-45% to 76-94% by addition of tri-leucine, and for netilmicin, whose ED values improved from 39-47% to 82-91%. The optimal relative amount of tri-leucine was determined for each of the three antibiotic powders and determined to be approximately 25%, i.e., optimal ED values were observed for powders containing 25% by weight tri-leucine relative to antibiotic.

Example 6
Aerosol Properties of Powders Containing Interferon-β in Combination with Trileucine

The broad applicability of the use of surface active di- and tri-peptides for increasing powder dispersivity was further explored in interferon-β powders. Interferon-β (a type I interferon) is a cytokine with antiviral, antiproliferative, and immunomodulatory activity.

Powders containing interferon-β and optionally tri-leucine and/or other excipients (hydroxyethylstarch, HES and raffinose) were prepared as described above. The solids content of the pre-dried solutions was 1%, with the exception of Lot No. RB27, which possessed a solids content of 0.5%. The composition of the final powders is given in Table 14 below.

Table 14. Interferon-β Powders Containing Tri-leucine

Lot #	Comp.	ED, % mean (n=10)	RSD, %	MMAD, μm	FPD, %	Yield, %	% < 5 μm, %
RB19	10% IFN-β 45%Leu-3 45% HES	81	7	3.2	48	56	79
RB21	10% IFN-β 45% Leu-3 45% Raff.	80	6	2.9	46	61	85
RB24	10% IFN-β 90% Leu-3	-	-	-	-	9*	-
RB27	10% IFN-β 67.5% Leu-3 22.5% Raff.	74	4	2.9	49	40	81
RB29	10% IFN-β 45% Leu-3 45% HES	79	5	3.2	41	50	83
RB36	10% IFN-β 22.5% Leu-3 67.5% Raff.	87	3	-	-	61	-
99320	10% IFN-β 90% Raff.	64	-	-	-	-	-

*No tests performed due to low yield.

As with the other active-agent containing powders, the addition of tri-leucine to powders composed of interferon-β served to increase the dispersivity and overall

aerosol properties of the resulting powder. Although the improvement was not as striking in some of the previous examples, addition of tri-leucine enhanced the ED values of an interferon-β powder from 64% to 74-87%. As in the previous example, it appears that an optimal amount of tri-leucine is around about 22-25% by weight for the IFN-β powder.

Example 7
Factor IX Dry Powders

Powders containing factor IX, a 55,000 dalton glycoprotein with a modular domain structure and numerous posttranslational modifications, useful in the treatment of hemophilia B, and trileucine and/or other excipient(s), were prepared to further explore the dispersivity-enhancing effects of tri-leucine and other surface active di- and tri-peptides on different medicaments.

Powders containing Factor IX, both with and without leucine or a leucine-containing dimer or trimer, were prepared as described previously. The solids content of the pre-spray-dried solution was 1 % by weight (w/v). Yields of the spray dried powders ranged from 40 to 60%. The formulations of the dried powders are provided in Table 15 below.

Table 15. Factor IX Powders

Formulation	Emitted Dose (RSD)	MMAD
93% Factor IX/7% NaCitrate	57 (5 %)	-
37% Factor IX/3% Na Citrate/60% Leucine	78 (3%)	2.9
56% Factor IX/4% Na Citrate/40% Trileucine	89 (5%)	2.7

The results in Table 15 further support the effectiveness of tri-leucine at significantly improving the dispersibility of dry powder compositions, irrespective of the active agent contained in the composition. Moreover, as in the previous examples, tri-leucine is better than leucine in significantly improving the dispersibility of the composition (from an ED of 57% to 89%), and can achieve such enhancement when used in smaller quantities than leucine.

Example 8
Stability Studies

The chemical and physical stability of packaged PTH powders under accelerated stability conditions were evaluated on the basis of the change in protein concentration and aerosol properties measured between initial and 3-month time points. PTH-trileucine and PTH-leucine powders were prepared as in Example 2 above.

Powders were hand-filled in blister packs (BPs). The blister packs were placed in petri dishes (20-60 BPs/dish).

Table 16. Accelerated stability study at 40 °C/Ambient Relative Humidity

Formulation ID Composition	Packaged Storage Condition no 2nd wrap no dessicant 40°C/ambient RH	%Purity (by area)	% Emitted Dose (rsd)	Fine Particle Dose (FPD) <3.3µm	MMAD (µm)	% Wt. Change (TGA)
R99484 30% PTH/ 70%Leucine	initial	97.0	79.6 (3)	0.58	2.5	1.4
	4 weeks	n/a	74.9 (5)	n/a	n/a	1.7
	6 weeks	n/a	75.2 (6)	n/a	n/a	n/a
	8 weeks	95.2*	78.8 (6)	0.55	2.4	2.2
	12 weeks	n/a	78.6 (3)	n/a	n/a	TBD
R99485 30% PTH/ 70% tri-leucine	initial	97.1	79.4	0.45	2.9	2.6
	4 weeks	n/a	75.8	n/a	n/a	2.4
	6 weeks	n/a	81.6	n/a	n/a	n/a
	8 weeks	94.8	81.6	0.44	2.9	2.4
	12 weeks	n/a	out of BP	n/a	out of BP	out of BP

* the chemical stability of the 8 weeks, 40°C/ambient RH sample is similar to the stability of the 6 months, 40°C/dry sample (foiled wrapped w/ desiccants) of a 30% PTH/70% mannitol formulation.

In looking at the results in Table 16, it can be seen that the trileucine-containing formulation is both chemically and physically stable upon storage, even at temperatures increased over ambient. Specifically, the 30% PTH/70% trileucine powder exhibited minimal degradation of protein over the timecourse of 3 months, while the aerosol performance of the powder remained essentially unchanged.

Example 9
Electron Spectroscopy of Chemical Analysis (ESCA) of Powder Formulations

ESCA analysis was carried out on certain powder formulations to investigate the surface enrichment of di-leucyl di- or tripeptide in the particles. The relative concentrations of powder components in the bulk powder is provided in the column, "Formulation"; the concentration of each component on the surface of the particles, as determined by ESCA, is provided in the column, "ESCA result".

Table 17. SCal/Raffinose/Leu formulations pH7

Lot No.		Formulation (%w/w)	ESCA result (%w/w)
R99282	sCal	5	53
pH7	Leucine	0	-
	Raffinose	95	47
R99283	sCal	5	11
pH7	Leucine	5	52
	Raffinose	90	38
R99284	sCal	5	39
pH7	Leucine	20	28
	Raffinose	75	33
R99286	Scal	5	26
pH7	Leucine	80	64
	Raffinose	15	9

Table 18. Leucyl-Peptide/Raffinose formulations

Lot No.		Formulation (%w/w)	ESCA result (%w/w)
R99337	Leucine-2	5	28.7
pH7	Raffinose	95	71.3
R99338	Leucine-2	20	44.1
pH7	Raffinose	80	55.9
R99339	Leucine-2	60	94.9
pH7	Raffinose	40	5.1
R99340	Leucine-3	20	97.1
pH7	Raffinose	80	2.9
R99342	Alanine-3	20	41.3
pH7	Raffinose	80	58.7

Table 19. SCal/Raffinose/Leu-3 formulations pH4

Lot No.		Formulation (%w/w)	ESCA result (%w/w)
R99435 pH4	Scal	5	36.6
	Leucine-3	0	0
	Raffinose	95	63.4
	Scal	5	17.9
pH4	Leucine-3	5	7.0
	Raffinose	90	75.1
R99437	Scal	5	46.4
pH4	Leucine-3	20	24.2
	Raffinose	75	29.4
R99438 pH4	Scal	5	22.7
	Leucine-3	40	74.8
	Raffinose	55	2.5
R99439 pH4	Scal	5	16.4
	Leucine-3	60	81.6
	Raffinose	35	2.0

The above results indicate that powders containing a surface active material are enriched at the surface in concentration of surface active material. Surface enrichment of di- or trileucine is observed for both the non-active agent containing powders in Table 18 and for the s. calcitonin powders in Table 19.

Although the ESCA results for the calcitonin powders are subject to some variability (this is due to the difficulty of separating out surface concentration contributions by components having the same atom within their structures i.e., calcitonin vs. trileucine), the overall trend observed supports the finding of powders in which the surface concentration of the di-leucyl di- or tripeptide is greater than that in the bulk powder.

IT IS CLAIMED:

1. A dry powder composition comprising an active agent and a di- or tripeptide comprising at least two leucines.

2. The dry powder composition of claim 1, wherein said composition is suitable for delivery to the lung or deep lung by inhalation.

3. The composition of claim 1 comprising dry powder particles, wherein the concentration of said di- or tri-peptide on the surface of the particles is greater than in the bulk powder.

4. The composition of claim 1, wherein said di- or tri-peptide is present in an amount effective to increase the emitted dose of the composition over the emitted dose of the composition absent said di- or tripeptide.

5. The composition of claim 1, comprising from about 1% by weight to about 99% by weight di- or tripeptide.

6. The composition of claim 5, comprising from about 5% to about 75% by weight di- or tripeptide.

7. The composition of claim 6, comprising from about 5% to about 50% by weight di- or tripeptide.

8. The composition of claim 1, further comprising a pharmaceutically acceptable excipient or carrier.

9. The composition of claim 8, wherein said excipient is selected from the group consisting of carbohydrates, amino acids, peptides, proteins, organic acid salts, and polymers.

10. The composition of claim 1, characterized by an emitted dose of at least about 30%.

11. The composition of claim 1, characterized by an emitted dose of at least about 45%.

12. The composition of claim 1, comprising a dipeptide where said dipeptide is dileucine.

13. The composition of claim 1, comprising a tripeptide comprising two leucines and an amino acid selected from the group consisting of leucine (leu), valine (val), isoleucine (isoleu), tryptophan (try) alanine (ala), methionine (met), phenylalanine (phe), tyrosine (tyr), histidine (his), and proline (pro).

14. The composition of claim 13, wherein said tri-peptide is trileucine.

15. The composition of claim 1, comprising particles having an MMD of less than about 10 microns.

16. The composition of claim 1, comprising particles having an MMD of less than about 4.0 microns.

17. The composition of claim 1, comprising particles having an MMAD of less than about 10 microns.

18. The composition of claim 1, comprising particles having an MMAD of less than about 4 microns.

19. The composition of claim 1, wherein the di- or tri-peptide possesses a glass transition temperature greater than about 40 °C.

20. The dry powder composition of claim 1 having stable dispersivity over time, as characterized by a drop in emitted dose of no more than about 10% when said composition is stored under ambient conditions for a period of three months.

21. The dry powder composition of claim 1 having chemical stability over time, as characterized by degradation of less than about 5% by weight of the active agent upon storage of said composition under ambient conditions for a period of three months.

22. The dry powder composition of claim 1, wherein said active agent is selected from the group consisting of insulin, cyclosporin, parathyroid hormone, follicle stimulating hormone, VLA-4 inhibitors, interleukin-4R, thrombopoietin, c-peptide, amylin, pro-insulin, interleukin-1, interleukin-2, alpha-1-antitrypsin, budesonide, human growth hormone, growth hormone releasing hormone, interferon alpha, interferon beta, growth colony stimulating factor, keratinocyte growth factor, glial growth factor, tumor necrosis factor, leutinizing hormone releasing hormone, calcitonin, low molecular weight heparin, somatostatin, respiratory syncytial virus antibody, erythropoietin, Factor VIII, Factor IX, ceredase, cerezyme and analogues, agonists and antagonists thereof.

23. The dry powder composition of claim 1, comprising particles having a bulk density from 0.1 to 10 grams per cubic centimeter.

24. The dry powder composition of claim 23, comprising particles having a bulk density from 0.5 to 2.0 grams per cubic centimeter.

25. A method for enhancing the aerosol performance of a dry powder, said method comprising:

incorporating into a liquid formulation comprising an active agent, a di- or tri-peptide comprising at least two leucines, and

drying said liquid formulation such that a dry powder containing the active agent and the di- or tri-peptide is produced,

whereby the resultant dry powder possesses an emitted dose that is increased over the emitted dose of a dry powder having the same components but absent said di- or tri-peptide.

26. The method of claim 25, wherein said incorporating step comprises incorporating a dipeptide where the dipeptide is dileucine.

27. The method of claim 25, wherein said incorporating step comprises incorporating a tri-peptide comprising two leucines and an amino acid selected from the group consisting of leucine (leu), valine (val), isoleucine (isoleu), tryptophan (try) alanine (ala), methionine (met), phenylalanine (phe), tyrosine (tyr), histidine (his), and proline (pro).

28. The method of claim 25, wherein said liquid formulation is an aqueous formulation.

29. The method of claim 25, wherein a pharmaceutically acceptable excipient or carrier is also incorporated into said liquid formulation.

30. The method of claim 25, wherein said drying step is selected from the group consisting of spray-drying, freeze-drying, and spray-freeze drying.

31. The method of claim 25, wherein the emitted dose of the dry powder is increased by at least about 5% over that of a dry powder having the same components and absent said di- or tri-peptide.

32. The method of claim 31, wherein the emitted dose of the dry powder is increased by at least about 10% over that of a dry powder having the same components and absent said di- or tripeptide.

33. The method of claim 25, wherein said dry powder is characterized by an emitted dose of at least about 30%.

34. The method of claim 25, wherein said active agent is selected from the group consisting of insulin, cyclosporin, parathyroid hormone, follicle stimulating hormone, VLA-4 inhibitors, interleukin-4R, thrombopoietin, c-peptide, amylin, pro-insulin, interleukin-1, interleukin-2, alpha-1-antitrypsin, budesonide, human growth hormone, growth hormone releasing hormone, interferon alpha, interferon beta, growth colony stimulating factor, keratinocyte growth factor, glial growth factor, tumor necrosis factor, leutinizing hormone releasing hormone, calcitonin, low molecular weight heparin, somatostatin, respiratory syncytial virus antibody, erythropoietin, Factor VIII, Factor IX, ceredase, cerezyme and analogues, agonists and antagonists thereof.

35. A method for increasing the aerosol performance of an active agent-containing formulation suitable for administration to the lung, said method comprising:

incorporating a di- or tripeptide comprising at least two leucines into a formulation comprising an active agent, to thereby form a composition comprising said active agent and said di- or tripeptide,

whereby as a result of said incorporating, the emitted dose of the composition is increased over the emitted dose of a composition having the same components but absent said di- or tripeptide.

36. The method of claim 35, wherein said composition is a liquid composition suitable for aerosolized administration to the lung.

37. The method of claim 35, wherein said composition is a dry composition suitable for aerosolized administration to the lung.

38. The method of claim 35, wherein said tripeptide comprising two leucines and an amino acid selected from the group consisting of leucine (leu), valine (val), isoleucine (isoleu), tryptophan (try) alanine (ala), methionine (met), phenylalanine (phe), tyrosine (tyr), histidine (his), and proline (pro).

39. A method for delivery of a dry powder composition to the lungs of a mammalian patient, said method comprising administering by inhalation the dry powder composition of claim 1 in aerosolized form.

40. A method for delivery of an active-agent containing formulation to the lungs of a mammalian patient, said method comprising administering by inhalation a liquid composition produced by the method of claim 36.

INTERNATIONAL SEARCH REPORT

Inter. nal Application No
PCT/US 00/09785

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/12 A61K47/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 21461 A (NOVONORDISK AS) 18 July 1996 (1996-07-18) page 7, line 1 - line 16 page 11 -page 12; example 1	1-11,13, 19-21, 25, 27-33, 35-38
A	US 5 182 258 A (CHIOU GEORGE C Y) 26 January 1993 (1993-01-26) column 9 -column 12; example 1	1-40
A	WO 97 03649 A (CO ORDINATED DRUG DEV LTD ;STANIFORTH JOHN NICHOLAS (GB)) 6 February 1997 (1997-02-06) page 12, line 9 - line 18 page 18 -page 19; examples 1-3	1-40

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 November 2000	30/11/2000

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Inter nal Application No
PCT/US 00/09785

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9621461 A	18-07-1996	US 5705482 A	06-01-1998
		AU 4329696 A	31-07-1996
		EP 0801573 A	22-10-1997
		JP 10511965 T	17-11-1998
		ZA 9600124 A	13-08-1996
US 5182258 A	26-01-1993	US 5283236 A	01-02-1994
		US 5278142 A	11-01-1994
WO 9703649 A	06-02-1997	AU 6620396 A	18-02-1997
		CA 2226657 A	06-02-1997
		EP 0871430 A	21-10-1998
		JP 11509546 T	24-08-1999

US006280729B1

(12) **United States Patent**
Huang et al.

(10) **Patent No.: US 6,280,729 B1**
(45) **Date of Patent: *Aug. 28, 2001**

(54) **PREPARATION OF FACTOR IX**

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(73) Assignee: **Aventis Behring LLC**, King of Prussia, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/524,206**

(22) Filed: **Mar. 13, 2000**

Related U.S. Application Data

(62) Division of application No. 08/465,867, filed on Jun. 6, 1995, now Pat. No. 6,043,215, which is a division of application No. 08/101,175, filed on Aug. 3, 1993, now Pat. No. 6,063,909, which is a continuation-in-part of application No. 07/662,927, filed on Mar. 1, 1991, now abandoned.

(51) **Int. Cl.**[7] **A61K 38/48**

(52) **U.S. Cl.** **424/94.64**; 514/8

(58) **Field of Search** 514/8, 381, 384; 424/94.64

(56) **References Cited**

U.S. PATENT DOCUMENTS

4,379,085		4/1983	Williams et al.	260/112 B
4,405,603		9/1983	Schwinn et al.	424/101
4,411,794		10/1983	Schwinn et al.	210/670
4,447,416		5/1984	Menache-Aronson	424/101
4,578,216		3/1986	Saundry	260/112 B
4,687,664		8/1987	Philapitsch et al.	424/85
4,725,673		2/1988	Herring	530/381
4,770,999		9/1988	Kaufman et al.	436/68
4,786,726		11/1988	Smith	530/381
4,981,952		1/1991	Yan	530/384
5,055,557		10/1991	Zimmerman	530/381
5,061,789		10/1991	Moller et al.	530/381
5,071,961		12/1991	Kraus et al.	530/384
5,281,661		1/1994	Linnau et al.	525/54.1
5,457,181		10/1995	Michalski et al.	530/381
5,614,500		3/1997	Zimmerman	514/21
5,639,857		6/1997	Zimmerman	530/384
6,043,215	*	3/2000	Huang et al.	514/8
6,063,909	*	3/2000	Huang et al.	530/412

FOREIGN PATENT DOCUMENTS

0 118 256	9/1984	(EP) .
0251874A1	1/1988	(EP) .
2080312A	2/1982	(GB) .
2080312B	12/1983	(GB) .
1258700A	10/1989	(JP) .

OTHER PUBLICATIONS

Pool, J.G. et al., *Nature*, 203, 312 (1964).
Kingdon H.S. et al., *Thromb. Diath. Haemorr.*, 33, 617–631(1975).
Miletech, J.P. et al., *J. of Biol. Chem.*, 253(19), 6908–6916 (1978).
Rosenberg et al., *J. Biol. Chem.*, 250, 1607–1617 (1974).
Cash, J.D. et al., *Vox Sang*, 35, 105–110 (1978).
Giles, A.R. et al., *Thrombosis Res.*, 17, 353–366 (1980).
Wessler et al., *J. Applied Physiol.*, 14, 943–946 (1959).
Pasternack et al., *Nature*, 322, 740 (1986).
Laemli, U.K., *Nature*, 227, 680–685 (1970).
Weber, K. et al., *J. Biol. Chem.*, 244, 4406–4412 (1969).
Bloom, J.W. et al., *Biochemistry*, 18, 4419–4425 (1979).
Jallat, S. et al., *EMBO Journal*, 9(10), 3295–3301 (1990).
Anson, D.S. et al., *Nature*, 315:683–685 (1985).
Blatt, P.M. et al., *Annals of Internal Medicine*, 81, 766–770 (1974).
Menache, D. et al., *Blood*, 64(6), 1220–1227 (1984).
Michalski et al., *Vox Sang*, 55, 202–210 (1988).
Goodall, A.H. et al., *Blood*, 59(3), 664–670 (1982).
Liebman, H.A. et al., *Blood*, 62(5), supp. 1, 288a (1983).
Liebman, H.A. et al., *PNAS*, 82, 3879–3883 (1985).
Bessos et al., *Thromb. and Haemos.*, 56(1), 86–89 (1986).
Bajaj, S.P. et al., *Preparative Biochemistry*, 11(4), 397–412 (1981).
Miletech, J.P. et al., *Methods in Enzymology*, 80, 221–228 (1981).
Tharakan, J. et al., *Vox Sang*, 58, 21–29 (1990).
Shapiro, S.S. et al., *Thromb. Diath. Haemorr.*, 16, 469 (1966).
Smith, K.J. et al., *Blood*, 72, 1269–1277 (1988).
Jenny, R. et al., *Preparative Biochemistry*, 16, 227–245 (1986).
Osterud, B. et al., *J. Biol. Chem.*, 253(17), 5946–5951 (1978).
Varadi, K. e al., *Thromb. Haemos.*, 35, 576–585 (1976).
Koehler G. and Milstein, C., *Nature*, 256, 495–497 (1975).
Smith, K.J. et al., *Thromb. Haemostas.*, 58, 349 (1987).
Brown, J.P. et al., *J. Biol. Chem.*, 225, 4980–4983 (1980).
Smith, K.J. et al., *Thrombosis Research*, 33, 211–224 (1984).
Burnouf et al., *Vox Sang.*, 57, 225–232 (1989).
Ahmad et al., *Thrombosis Research*, 55, 121–133 (1989).

* cited by examiner

Primary Examiner—Karen Cochrane Carlson
(74) *Attorney, Agent, or Firm*—Synnestvedt & Lechner LLP

(57) **ABSTRACT**

A novel method of protecting blood coagulation factor IX from proteases during purification or storage is disclosed. High concentrations of one or more water soluble organic or inorganic salts are used to stabilize factor IX, contained within blood plasma-derived solutions, or contained within solutions derived from other sources, against conversion to clinically unacceptable peptide structures such as factor IX_α, and/or degraded factor IX peptides. The technique is useful in stabilizing intermediate purity factor IX preparations during purification, and in maintaining the integrity of purified factor IX during long term storage. Stable high specific activity factor IX preparations are also disclosed.

9 Claims, 4 Drawing Sheets

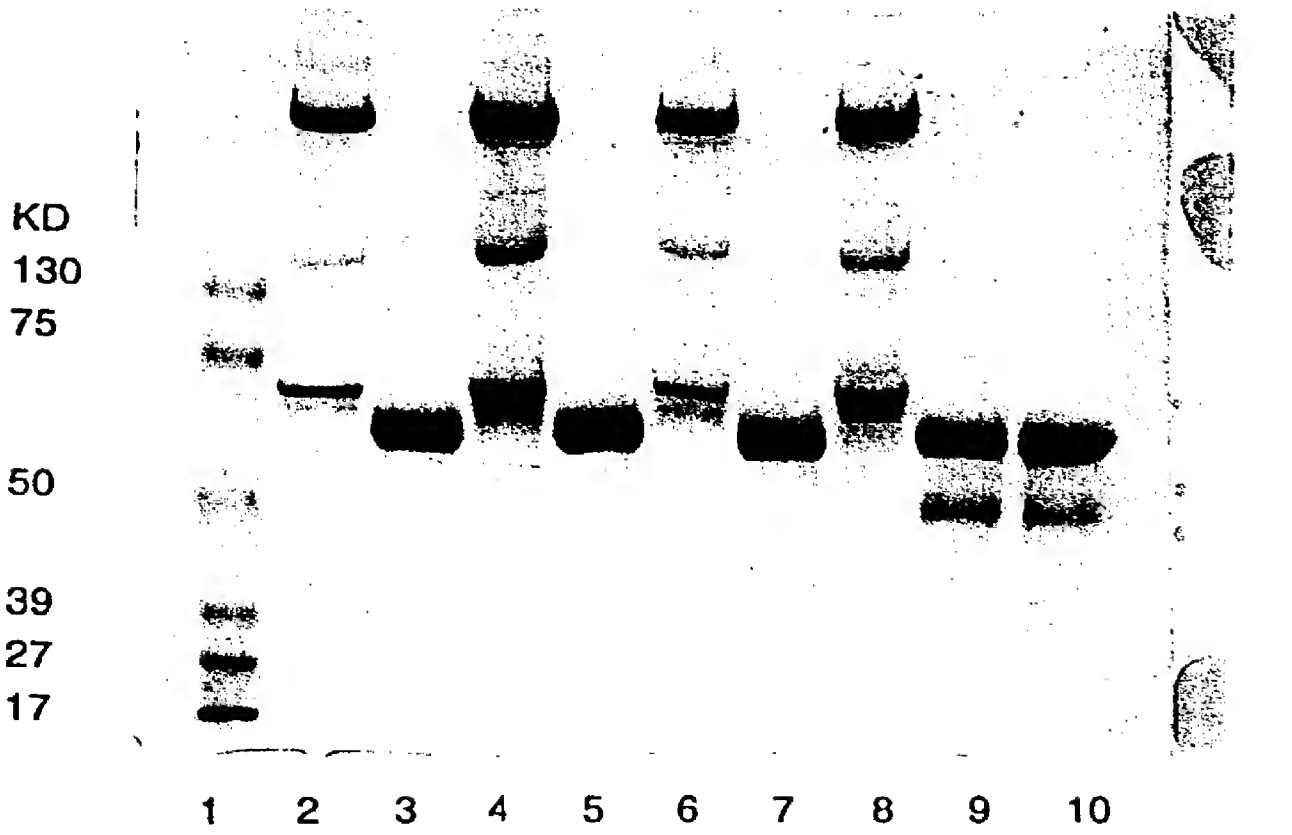

FIG. 1

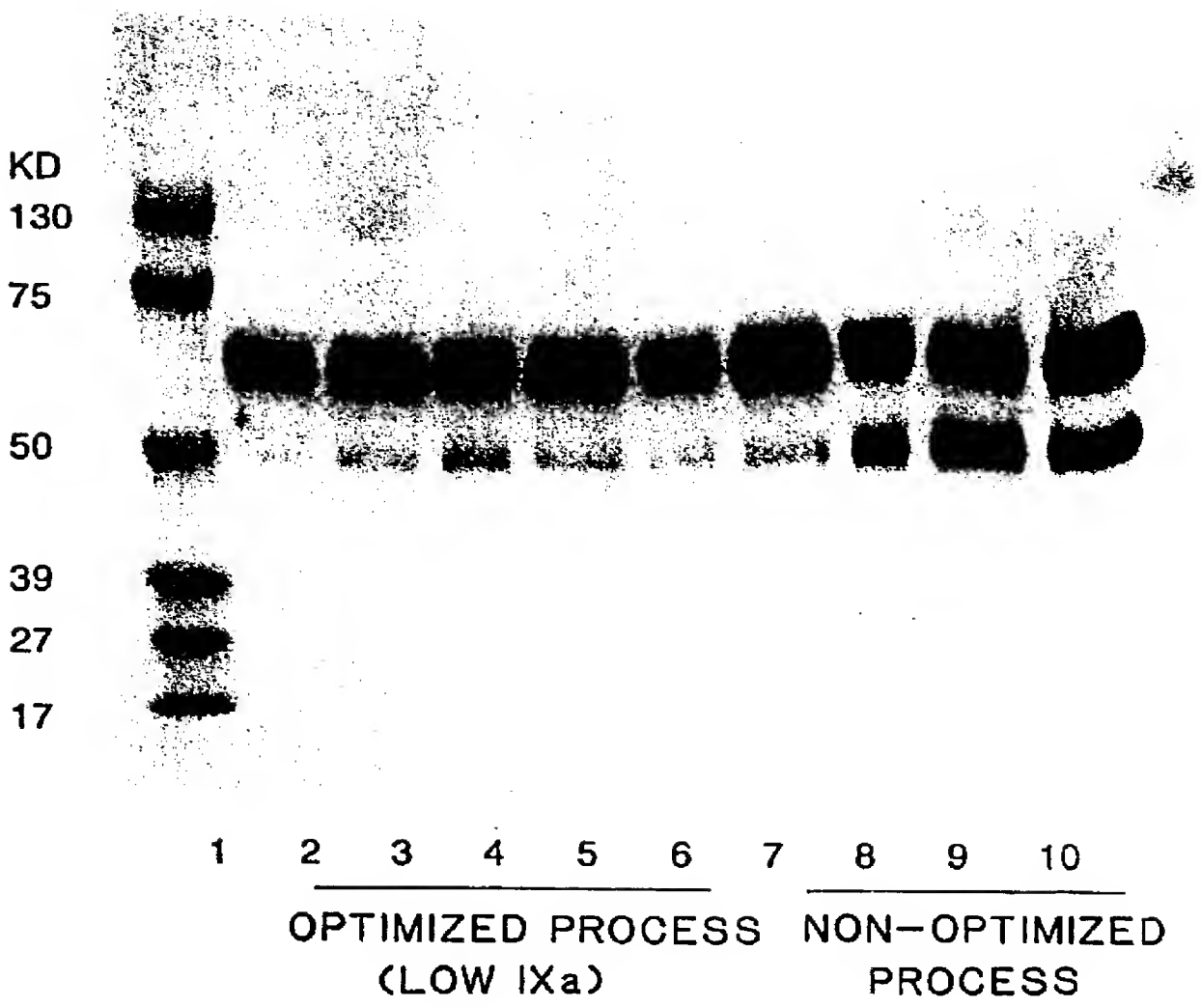
KD
130
75
50
39
27
17
1
2
3
4
5
6
7
8
9
10
OPTIMIZED PROCESS
(LOW IXa)
NON-OPTIMIZED
PROCESS

FIG. 2

FIG. 3

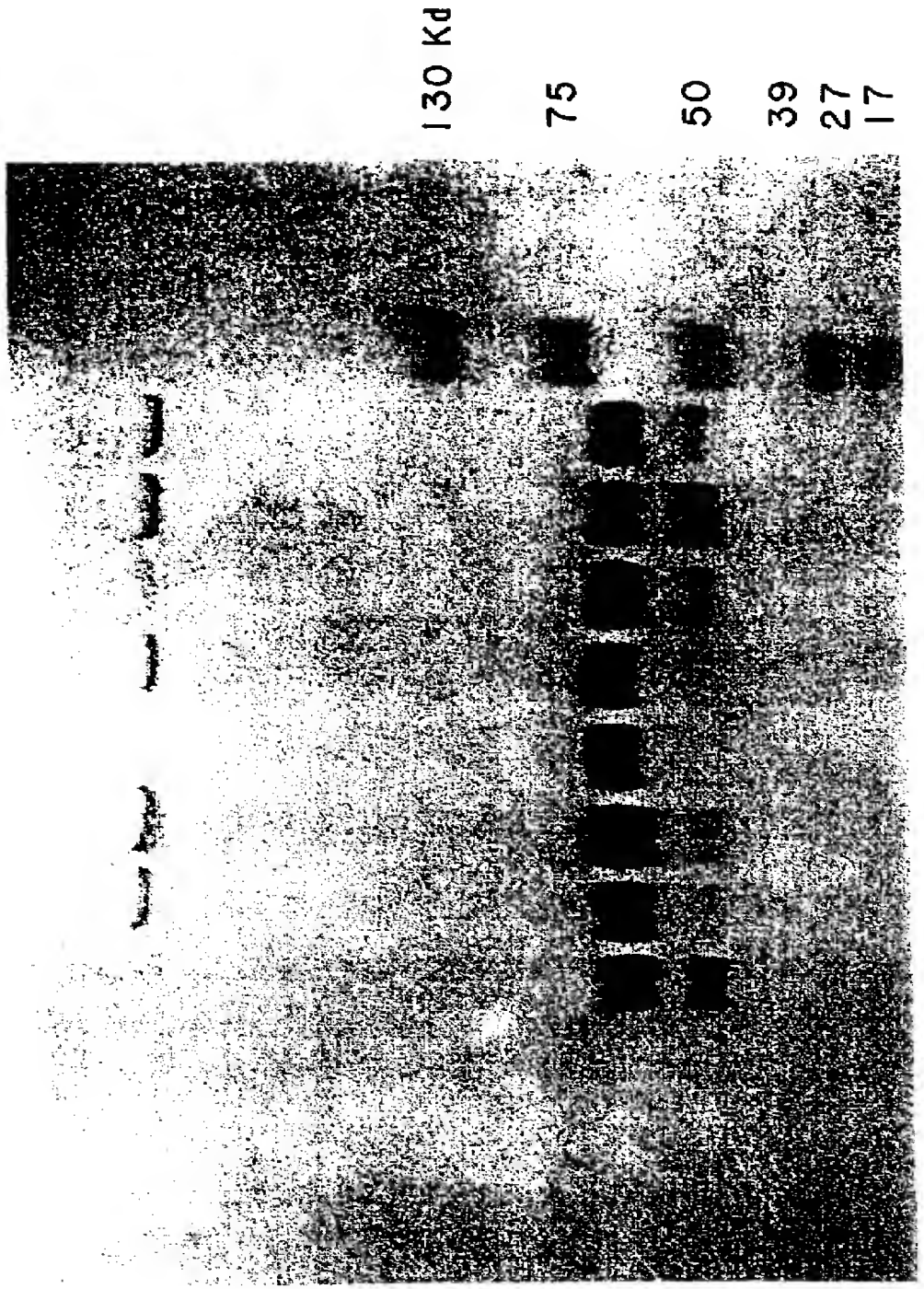

FIG. 4

PREPARATION OF FACTOR IX

RELATED APPLICATIONS

This application is a division of U.S. application Ser. No. 08/465,867, filed Jun. 6, 1995 now U.S. Pat. No. 6,043,215, which is a division of U.S. application Ser. No. 08/101,175, filed Aug. 3, 1993 now U.S. Pat. No. 6,063,909 which is a continuation-in-part of U.S. application Ser. No. 07/662,927, filed Mar. 1, 1991, now abandoned, all of which are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to purifying and stabilizing factor IX, one of the proteins essential to the cascade of reactions which accomplishes blood coagulation at the site of a wound.

Factor IX is a globular protein which has a molecular weight of about 70,000 daltons and which, in a normal individual, is constantly produced in the liver and circulates at a normal blood plasma concentration of approximately 5 μg/ml.

Hemophilia B (also known as Christmas disease) is a very serious illness which results in decreased in vivo and in vitro clotting activity and requires extensive medical monitoring throughout the life of the affected person. Such persons show normal clotting times only when provided with exogenous factor IX which is extracted from the blood plasma of normal individuals. Except for such treatment, the afflicted person can suffer from spontaneous bleeds in joints which produce severe pain and debilitating immobility, bleeds into muscles resulting in large volumes of blood accumulating in the tissue, spontaneous bleeds in the throat and neck which may cause asphyxiation if not immediately treated, bleeding into the urine, and severe bleeding following surgery or minor accidental injuries or dental extractions.

Functional factor IX deficiencies can arise in different ways. The gene coding for factor IX is located on the X chromosome. This explains why hemophilia B is much more common in males than females. Some of the afflicted persons are known to have inherited an X chromosome with a complete deletion of the factor IX gene. These severely affected persons may even produce antibodies to therapeutically injected factor IX. Many hemophilia B patients are known to produce a factor IX molecule with an altered amino acid sequence which results in molecules of partial or no coagulation activity. Some hemophilia B patients produce normal factor IX, but in insufficient quantities to effect clotting within a normal time after injury.

As mentioned above, Factor IX activity can be restored in the patient by injection of normal human plasma. However, at minimum, several liters would have to be administered to raise the patients's circulating factor IX levels to an effective range. Accordingly, the emphasis in therapy for hemophilia B patients has been to provide injections of a plasma concentrate highly enriched in factor IX. The provision of such a concentrate is no easy task, as will become apparent from the discussion which follows.

The mechanisms whereby circulating blood is generally prevented from clotting, yet directed to clot at the site of a wound are very complex and involve numerous proteins, other macromolecules, cells and structures. This hemostatic mechanism also utilizes numerous feedback or amplification pathways to further regulate coagulation. Owing to the large number of individual protein species which make up the clotting pathway and the large number of other macromolecules in blood plasma, it is generally difficult to isolate useable quantities of any one component, including factor IX, in highly pure form. In addition, blood contains numerous proteases (enzymes which digest or damage other proteins) which can affect adversely the protein selected for isolation, such as factor IX, before it can be separated from other blood components.

Since the clotting ability of the blood is held in a controlled balance, factor IX and other components associated with coagulation must be held inactive most of the time to avoid unnecessary clotting. Yet, the proteins must always be present throughout the circulatory system—ready to react immediately when needed.

The blood therefore contains by necessity a very complicated mechanism to prevent clotting from taking place where it is not needed, to clear unwanted clotting, and to rapidly stop the loss of blood at an injured site. The elucidation of this complicated mechanism of regulation makes clear why it is so difficult to isolate therapeutic factor IX free of clinically dangerous contaminants.

The formation of an effective clot involves the complex interaction of many vascular system components, including platelet blood cells, collagen and microfibrils exposed by damage to the vascular epithelium, phospholipids, and circulating proteins. Proteins which circulate in the blood as inert proenzymes and which are involved in coagulation are typically referred to as "coagulation factors". Upon activation, they generally function as highly specific enzymes which make specific alterations in other coagulation factor proenzymes. Thus, in turn, each sequential factor is activated. Some proenzymes, such as factor XII, may also be activated by contact with a damaged surface or by complexing with other macromolecules.

The mechanism of the clotting process is known in considerable detail. The active form of a coagulation factor is denoted by the subscript "a" and is typically produced from the inactive proenzyme by the action of another of the factor-specific proteases. In theory, administration of activated coagulation factors to hemophilia patients carries a risk of clot formation at many locations besides the site of injury.

The hemostatic mechanism may be characterized as a very delicate balance between those materials or processes which inhibit coagulation and those which enhance it. Oversupply of one or more substances, particularly activated coagulation factors, may lead to unwanted coagulation. Activated coagulation factors can therefore be dangerous contaminants in therapeutic preparations of coagulation proenzymes, such as factor IX preparations.

With respect to hemophilia B patients, the state of the art, however, involves their being typically treated with "prothrombin complex concentrate", which is a plasma extract concentrated in factor IX, but containing also significant amounts of other plasma proteins, including factors II, VII, X, active forms thereof, and numerous other contaminating proteases. Such preparations can also routinely be contaminated with factor IX_a.

There are numerous reports in the literature on the adverse clinical consequences of administering prothrombin complex concentrate (or other factor IX concentrates) contaminated with factor IX_a and/or with active or degraded forms of other clotting factors. The most serious risk is the inadvertent activation of the clotting cascade. Deaths have been documented.

Solutions to the problems associated with the use of impure factor IX concentrates have been hampered by lack

of understanding of exactly how and why such concentrates induce unwanted clotting. It has been proposed that factor IX concentrates may induce coagulation not only because amounts of factors IX_a are present, but also because they are significantly contaminated with other clotting factors, thus overloading the blood with high circulating levels of one or more clotting factors, or activated forms thereof.

Based on determinations using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), factor IX_a has an approximate molecular weight of 54,000. However, other peptide species which result from proteolytic degradation of factor IX have very similar molecular weights for example, from approximately 40,000 to approximately 65,000. It is not currently known whether factor IX activation products or degradation products are primarily responsible for adverse clinical consequences which have been observed on administration of prothrombin complex concentrate or other impure factor IX-containing concentrates. Consequently, the development of factor IX therapeutics which avoid the hazards of impure preparations is of great pharmaceutical interest.

The prior art discloses various strategies for the purification of factor IX, including the use of monoclonal antibody affinity technology to separate factor IX from other coagulation factors. However, it is important that such factor IX purifications include every effort to minimize the formation of factor IX_a or degraded factor IX peptides. Since factor IX (the proenzyme) and activated factor IX (IX_a) are similar in structure, it is difficult to separate the two forms of factor IX by any purification strategy. Even antibody affinity technology is unlikely to achieve a separation since both molecules have most of their potential determinants in common, and the factor IX_a contaminant (and potentially factor IX degradation peptides) would tend to be carried forward into the final product. It is believed that there is no presently known antibody of adequate differential sensitivity.

The present invention is related to improved means for preventing proteolytic conversion of factor IX to factor IX_a or to degraded factor IX peptides as factor IX is purified, and for arresting proteolytic action on purified factor IX during storage thereof.

REPORTED DEVELOPMENTS

The purification of factor IX requires that it be separated from numerous other blood plasma proteins and other plasma macromolecules. Typically factor IX is produced from cryoprecipitate-free plasma. This plasma fraction is produced by rapidly freezing whole plasma, allowing it to thaw slowly and collecting the separated supernatant. See Pool, J. G., et al. *Nature*, 203, 312 (1964). Many proteins, including factor VIII, precipitate out of the slowly thawing plasma and can be removed by centrifugation.

In the practice of the prior art, the factor IX-containing cryoprecipitate-free supernatant plasma is typically mixed with an anion exchange resin or gel such as DEAE Sephadex®, DEAE cellulose, or DEAE Sepharose®, (in batch or column form) or alternately $Al(OH)_3$ gel, leading to adsorption of the factor IX along with other clotting factors with similar binding properties, and also adsorption of lesser amounts of other contaminating proteins. Initial fractionation with anion exchange resin particles takes advantage of the fact that certain clotting factors (such as factors II, VII, IX and X) are selectively adsorbed onto such resins owing to their negatively charged gamma carboxyglutamate residues. These proteins are also referred to as "vitamin K-dependent clotting factors" since a posttranslational vitamin K cofactor-dependent process attaches the gamma carboxy groups, such groups being necessary for proper binding of the coagulation factors to Ca^{+2}, lipid surfaces and platelets.

Typically, the bound factor IX is washed, and then eluted from the anion exchange resin using a buffered salt solution of high molarity. Inasmuch as such high molarity salt solution is considered osmotically incompatible with human tissues, practitioners of the prior art invariably subject their factor IX extracts to dialysis and filtration (or alternately ultrafiltration and diafiltration) which place the factor IX extract in concentrated form, but replace the high molarity salt solution with a physiologically-compatible low molarity salt solution.

The factor IX-enriched resin eluate (contaminated with significant amounts of other proteins, including clotting factors II, VII and X) is known as a prothrombin complex concentrate. Factor II (prothrombin) and the other vitamin K-dependent coagulation factors are known as "prothrombin complex" because of the above-mentioned similar binding properties. Hemophilia B patients have been typically treated with such concentrates.

In the practice of the prior art, the above-mentioned anion exchange chromatography may also be preceded or replaced by other steps. For example, cryoprecipitate-free plasma, to which citrate has been added, can be treated with barium chloride causing precipitation of barium citrate on which factor IX and certain other coagulation factors are bound. The proteins are isolated from the precipitate and then subjected to anion exchange chromatography. See, for example, Miletich, J. P. et al., *J. Biol. Chem.*, 253(19), 6908–6916 (1978). Factor IX may also be adsorbed to a gel of $Al(OH)_3$.

Alternatively, a second ion exchange resin step can be added. As disclosed in U.S. Pat. No. 4,447,416 (hereinafter the '416 patent), after anion exchange chromatography, the factor IX fraction is subjected to ultrafiltration and diafiltration against 0.15 Molar NaCl buffered with 20 mM citrate, pH6, and then subjected to a second phase of ion exchange chromatography using a sulfated dextran resin. Factor IX is eluted from this second resin when the salt concentration reaches 0.8 Molar. The factor IX, contained within a solution of high salt molarity, is then again subjected to ultrafiltration and diafiltration against physiologically acceptable 0.11 Molar NaCl, 20 mM citrate, pH 6.8, and then stored in lyophilized form. The method reported, however, leads to a factor IX product in which less than 10% of the protein is factor IX, more than 90% of the material consisting of contaminating protein species. No assays of factor IX_a are reported in the '416 patent and no experiments are disclosed which would determine whether contaminating proteases will continue to generate factor IX_a, or degraded forms of factor IX, if the product were to be stored in other than lyophilized form.

Menache, D. et al., in *Blood*, 64(6), 1220–1227 (1984), using technology reflecting the '416 patent disclose a factor IX preparation, stated to be free of activated factor IX_a and having a specific activity of approximately 5 units factor IX activity per mg total protein. Since the specific activity of pure factor IX is approximately 200 units/mg protein, the final product is considered to be heavily contaminated with other proteins, and because of the potential for protease activity, its long term non-thrombogenic status is doubtful.

Michalski et. al., *Vox Sang*, 55, 202–210 (1988) discloses a factor IX purifying strategy in which standard anion exchange chromatography of the prior art is followed by

chromatography on a resin coated with heparin, a negatively charged mucopolysaccharide. The product is stated to be free of factor IX_a, as measured by an antithrombin III neutralization assay, but contains only 5% by weight of factor IX, the remainder being protein contaminants. It is clinically undesirable to administer such impure preparations owing to the potential for eventual activation of factor IX by one of the contaminating proteins present therein. Without evaluation of such concentrate in animal models sensitive to thrombogenic components as described in this application, the absence of thrombogenic risk is not verified.

Since blood plasma contains many proteins which have similar physical properties and which are very difficult to separate from factor IX, and because most purification procedures result in considerable proteolytic activation and/or degradation of factor IX, practitioners have tried to develop new strategies to obtain factor IX therapeutics having high specific activity (high purity) and being free of other protein contaminants.

Considerable emphasis has been placed on cloning the human factor IX gene in order to produce factor IX preparations which are free of other clotting factors and blood plasma proteases which tend to degrade Factor IX. Unfortunately to date, it has not been possible to isolate factor IX from such preparations in adequately pure form. In addition the obstacle of duplicating, in the genetically engineered product, the specific in vivo post translational modification of particular glutamic acid residues to yield the critical gamma-carboxyglutamic acid residues of factor IX has not been solved.

Antibodies specific to individual proteins have been a valuable tool in attempting to isolate coagulation factors in pure form. Following the technique of Koehler, G. and Milstein, C. (*Nature*, 256, 495–497 (1975)), Goodall, A. H. et al. identified monoclonal antibodies to factor IX and used them in preparative immunoaffinity chromatography to create a high specific activity factor IX. *Blood*, 59 (3), 664–670, (1982). However, the Goodall procedure does not solve the crucial clinical difficulty that factor IX_a exists in native plasma and will be further produced by proteolytic activity during the preliminary steps of the protocol (conventional chromatography such as anion exchange) prior to immunoaffinity chromatography. No assays for factor IX_a or degraded factor IX peptides were reported. No strategy for controlling thrombogenicity prior to immunoaffinity chromatography was disclosed, nor were test results in animal models sensitive to thrombogenic components reported.

Monoclonal antibody affinity techniques are very effective at separating factor IX from other clotting factors and have become the preferred purifying method of many researchers. However, in the present state of the art, factor IX obtained by immunoaffinity chromatography is consistently contaminated with copurifying factor IX_a, and/or other clinically unacceptable degraded forms of factor IX, which cross react with factor IX antibodies.

U.S. Pat. No. 4,786,726 (hereinafter the '726 patent) discloses a particular monoclonal antibody therein designated as "A-7". The development consists of recognizing that the binding of this antibody to factor IX is Ca^{+2} dependent, and can be prevented by the addition of ethylene diaminetetracetic acid (EDTA). This provides a useful method of controlling elution of factor IX from the stationary phase of an affinity column following washing to remove protein contaminants. Factor IX is finally eluted from the antibody-resin complexes using calcium chelation. The development does not, however, improve the quality of the prothrombin complex concentrate applied to the immunoaffinity column, nor does it provide a way to separate factor IX from factor IX_a. No recognition is given of the necessity or means of controlling factor IX activation or degradation in early processing stages.

Smith, K. J. et al., *Thrombosis Research*, 33, 211–224 (1984), in a publication reflecting the '726 patent, failed in attempting to create an antibody which would recognize factor IX and not factor IX_a (or vice-versa). The current art discloses no such antibody (which, in fact, because of structural considerations is likely to be very difficult to isolate) emphasizing the need for other purifying steps and methods which will solve the problem of activation of factor IX to factor IX_a, or its degradation to other clinically unsafe peptides.

In order to produce the most clinically acceptable factor IX, free of thrombogenic components, it is necessary to control decomposition of, or activation of, factor IX throughout the preparative procedure and especially in early stages thereof. Lack of a solution to this problem is consistently noted by the prior art. For example, it has been reported that immunoaffinity purified factor IX product showed contamination by lower molecular weight components (including IX_a) attributed to factor IX decomposition in the starting material.

In addition, in reference to another immunoaffinity purification system, H. A. Liebman et al. also notes the same inability to differentiate and separate factors IX and IX_a. *Blood*, 62(5), supp. 1, 288a (1983). See also Liebman, H. A. et al., *Proc. Natl. Acad. Sci. USA*, 82, 3879–3883, (1985).

The immunopurification system of H. Bessos et al. is reported to have generated a factor IX of high specific activity whose activity rapidly decayed after purification when the product was placed in a low ionic strength storage solution. *Thrombosis and Haemostasis*, 56(1), 86–89 (1986). This decay may have been caused by protease activity.

The prior art has also attempted, with only partial success, to prevent activation of factor IX to factor IX_a using particular protease inhibitors many of which are organic compounds typically of high toxicity. These compounds are suitable for in vitro research application only and are very undesirable as reagents in protocols for clinical products. For example, the '726 patent specifies that immunoaffinity chromatography of factor IX must take place in the presence of benzamidine. To obtain factor IX sufficiently pure and stable for the creation of specific monoclonal antibodies, Goodall, A. H. et al. (*Blood* 59 (3) 664–670 (1982)) teaches the addition of the toxic organics benzamidine and diisopropylfluoro-phosphonate to factor IX-containing solutions.

The present invention relates to stabilizing factor IX against activation or degradation.

SUMMARY OF THE INVENTION

In accordance with the present invention, there is provided, in a process for purifying and preserving factor IX derived from human blood plasma or other source, the improvement of stabilizing factor IX in solution against activation to factor IX_a or against degradation to peptides of altered length and/or conformation by adding one or more soluble organic or inorganic salts to the factor IX-containing solution to a concentration sufficient to prevent or substantially minimize activation of or degradation of factor IX but at a concentration insufficient to cause precipitation of, irreversible alterations in, or denaturation of the factor IX

molecule, and maintaining the salt(s) in the solution at said concentration during storage or further treatment.

In one respect, the present invention involves protecting factor IX from activation to factor IX_a or degradation to peptides of altered length and/or conformation during multi-step purification by minimizing the amount of time impure factor IX is present in solutions containing an insufficient concentration of salt. The improved processes of the invention are found to be particularly useful in preventing catalytic action by proteases upon factor IX. Accordingly, there is provided in a method of purifying factor IX in solution involving two or more sequential separation processes, the improvement which comprises adding, during or after a particular separation process, one or more soluble organic or inorganic salts to the solution containing factor IX to a concentration of salt sufficient to prevent or substantially minimize catalytic action by proteases upon factor IX, but at a total concentration of said salt or salts insufficient to cause precipitation of, irreversible alterations in, or denaturation of the factor IX molecule, and maintaining the partially purified protein in contact with said salt solution at said sufficient salt concentration for a period at least until the next separation process is commenced.

As will be described in detail below, a wide variety of water soluble organic and inorganic salts can be used in the practice of the invention. Preferred salts are water soluble alkali metal or alkaline earth metal salts, most preferably magnesium, potassium, sodium and lithium chloride or sodium sulfate. It is expected that the most widely used concentration of salt will fall within the range of about 0.4 to about 1.4 Molar.

Another aspect of the invention comprises an aqueous solution of partially purified factor IX having a predetermined specific activity and one or more water soluble organic or inorganic salts in a concentration sufficient to prevent or substantially minimize catalytic action by proteases upon factor IX, but insufficient to cause irreversible alterations in, precipitation of, or denaturation of the factor IX molecule, the solution when stored for at least about 12 hours at or below a temperature of 4° C., having also a factor IX activity of about 80 to about 100% of the predetermined activity.

A further aspect of the invention relates to the provision of a factor IX composition which is in therapeutic form and is stable in that it is capable of being stored as an aqueous solution without deteriorating for prolonged periods of time, for example, at least about 2 weeks at room temperature. Accordingly, the invention encompasses also a therapeutic composition comprising an aqueous solution of factor IX having a specific activity of greater than about 50 units factor IX activity/mg protein, and wherein the composition is capable of being stored for a period of time of about 2 weeks at a storage temperature up to 15–30° C., while remaining free of factor IX_a, degraded forms of factor IX and/or active forms of other clotting factors in concentrations which would tend to cause detectable adverse clinical effects in a human when said composition is administered in a therapeutic dose.

A further aspect of the invention provides a method of treating Christmas disease in a patient comprising administering to such patient an effective amount of one or more of the therapeutic compositions of this invention. As will be seen from the discussion below, a variety of inorganic and organic salts which are non-toxic to humans can be used in formulating such compositions in accordance with the present invention.

Important advantages afforded by the invention include: 1) improving the purity, safety and stability of factor IX therapeutics; and 2) introducing flexibility and economy into commercial factor IX production schedules. Still another advantage afforded by the invention is that stable highly purified factor IX can be produced without use of therapeutically undesirable and highly toxic organic protease inhibitors. In addition, the nature of the invention is such that it is widely applicable to many types of processes which have been developed for producing factor IX preparations, including those of low, intermediate or high purity.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is an SDS-polyacrylamide slab gel showing DEAE concentrates and final factor IX products produced therefrom by a typical prior art process or by a process of the present invention.

FIG. **2** is a Western blot showing DEAE concentrates and final factor IX products produced therefrom by a typical prior art process or by a process of the present invention.

FIG. **3** is a Western blot showing the levels of proteolysis of factor IX which occur at 25° C. in samples of factor IX-containing DEAE concentrate dialyzed against different molarities of sodium chloride.

FIG. **4** is a Western blot showing levels of proteolysis of factor IX which occur at 4° C. in samples of factor IX-containing DEAE concentrate dialyzed against 0.5 Molar concentrations of different salts.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon the recognition that water-soluble organic and inorganic salts can be used to protect factor IX from proteolysis, including activation to factor IX_a, during purification of factor IX. One of the important advantages of the present invention is that it can be used effectively in any number of different processes which are available for purifying factor IX. There follows a description of a basic type of process for producing purified concentrated factor IX.

The production of factor IX for therapeutic use in the treatment of hemophilia B (Christmas disease) traditionally begins with blood plasma which is subjected to freezing. This frozen plasma is then slowly thawed at which point clotting factor VIII and certain other proteins can be recovered as a cryoprecipitate. Factor IX and other proteins move into the soluble supernatant phase.

This factor IX-containing plasma fraction is then typically subjected to adsorption on an anion exchange resin. After washing the resin particles extensively with a dilute salt solution to remove unbound or weakly binding proteins, a high molarity salt solution is usually used to elute factor IX which is collected in a fraction known as the "prothrombin complex concentrate" because it also contains significant amounts of the other vitamin K-dependent or "prothrombin complex" clotting factors (factors II, VII and X, and also activated forms thereof).

Typically, this factor IX-enriched fraction is then subjected to a lengthy filter concentration and dialysis procedure which can take up to 24 hours for the purpose of replacing the high salt medium with a low salt molarity, physiologically compatible buffer. In earlier times, this form of the product was used for therapeutic purposes.

Typically, this low-salt form of prothrombin complex concentrate contained proteases which prematurely activate

factor IX to clinically dangerous factor IX_a. Under these circumstances, factor IX is also degraded to other peptides which may also be clinically dangerous. Such proteolysis of factor IX is particularly severe when the factor IX is stored for prolonged periods in salt solutions of low molarity. Deaths have been reported from the administration of this type of product. Plat, P. M. et al., *Annals of Internal Medicine*, 81, 766–770 (1974).

In an attempt to improve on the clinically inadequate purity of prothrombin complex concentrate, researchers have modified the basic process described above, for example, by adding various additional steps or separation procedures, or by adopting alternate strategies to prevent proteolysis of factor IX and to remove additional contaminants. More recent protocols are typically a variation on the following: 1) cryoprecipitation; 2) anion exchange which takes advantage of the common specific adsorbability of vitamin K-dependent clotting factors; and 3) an additional separation procedure which separates factor IX from the other prothrombin complex proteins.

Examples of such additional separation procedures include the use of chromatography on an agarose gel to which heparin groups have been attached, cation exchange on a sulfated dextran gel, or immunoaffinity chromatography in which the stationary phase of the separation (purification) system consists of factor IX-specific antibodies.

Another strategy involves use of ammonium sulfate fractionation and/or elution of factor IX from an adsorption complex formed from precipitated barium salts followed by anion exchange chromatography. Selective adsorption to a gel of aluminum hydroxide has also been utilized. Still another strategy involves the use of an additional anion exchange resin which effects separation of prothrombin complex proteins which were not separated when previously contacted with different type anion exchange resin.

Still another approach is to apply whole plasma or cryoprecipitate-free plasma directly to a factor IX-specific immunoaffinity chromatography resin present in batch or column form.

Available methods for purifying factor IX, such as those described above, involve separation processes and manipulations. The term "separation process," as used herein, refers to a step which involves separating factor IX from one or more other peptides (proteins) in admixture therewith. The term "manipulation," as used herein, refers to a step which does not effect separation of factor IX from other peptides (proteins), but which is practiced before or after a separation process. Examples of manipulations are dialysis, sterile filtration, heat sterilization to inactivate contaminating microorganisms or virus particles in factor IX solutions, and also diafiltration wherein factor IX is selectively retained against a selectively permeable membrane while the solvent in which the factor IX is suspended is filtered through and replaced.

When a separation process or a manipulation of the prior art requires use of a salt, it is routinely performed using a low molarity salt solution (generally 0.15 M), except when the nature of the process or the manipulation requires that it be performed with the use of a high salt concentration. In the latter case, it is conventional to lower the salt concentration promptly after the step involving its use is completed and without eaffecting storage of factor IX therein. Examples of manipulations wherein a high salt concentration not intended to protect factor IX from proteolysis is removed promptly after the step involving its use include (1) eluting factor IX from a chromatography column using a high salt buffer and then removing the salt by procedures such as dialysis or by freezing and lyophilizing the eluate, thereby removing the salt by volatilization, or (2) heating a factor IX-containing solution to inactivate microorganisms or viruses. In the course of the development of this invention, it has been recognized that reducing the concentration of organic or inorganic salt in a factor IX-containing solution during or following completion of a separation or manipulation procedure provides a major opportunity for activation of factor IX to factor IX_a or for degradation of factor IX to other factor IX-derived peptides. It is believed that there are two general reasons that the significance of proteolysis of factor IX and resultant production of factor IX_a in low salt environments has gone unrecognized in the art.

First, high molarity salt solutions interfere with certain types of separation processes. For example, high concentrations of salts would prevent binding by factor IX to certain ion exchange resins or immunoaffinity columns. No separation could be effected.

Second, factor IX-containing high molarity salt solutions cannot be injected into patients because they are osmotically incompatible with living tissue. As a result, the prior art regards "excess" salt as something which should be removed from a factor IX preparation at the earliest convenience.

The use of salts at relatively high concentrations in accordance with the present invention enables factor IX to be protected not only during manipulations between or after separation processes, but during the separation processes themselves. The use of the "high" salt concentration in accordance with the present invention can be employed, as appropriate, in the various steps utilized in the purifying process up to and including that stage of the purification at which there have been removed from the Factor IX-containing solution those impurities which tend to activate Factor IX to Factor IX_a and/or to degrade Factor IX to peptides of altered length and/or conformation. If desired, the high salt concentration can then be reduced. In the preferred embodiment of the present invention, the high salt concentration (for example, about 1 Molar or more) can be reduced somewhat (for example, to about 0.4 to about 0.6 Molar) just prior to the purifying step which involves immunoaffinity chromatography using monoclonal antibodies which effect separation of Factor IX from other coagulation factors and other proteins. Thereafter, the Factor IX can be eluted from the monoclonal antibodies and recovered in dilute form, for example, in a dilute solution having a Molarity below about 0.4.

The following publications disclose examples of factor IX purifications which can be modified in accordance with the present invention for the purpose of producing a therapeutic material of higher purity, ard improved safety and stability. In one exemplary embodiment of such modifications, there is included and maintained in the impure factor IX solution a soluble salt (for example, potassium chloride) in a relatively high concentration, for example, at least about 0.5 Molar.

(A) U.S. Pat. No. 4,447,416 discloses a process for purifying factor IX utilizing an anion exchange resin in a low molarity salt solution with the steps of clarification and concentration by filtering at low salt concentration following. After further purification on a sulfated dextran cation exchange resin, the solution is dialyzed against a low molarity salt solution.

(B) Michalski et al. *Vox. Sang.*, 55, 202–210 (1988) disclose a factor IX product purified by traditional

anion exchange chromatography and the subsequent use of a heparin-linked resin. Numerous low ionic strength procedures are involved such as elution from the anion exchange resin with NaCl buffer, dilution prior to binding to the heparin-coated resin, and elution therefrom.

(C) Miletich J. P. et al. in *Methods in Enzymolocry*, 80, 221–228 (1981) disclose a factor IX product derived from successive steps of barium citrate adsorption, ammonium sulfate fractionation, ion exchange and dextran sulfate chromatography (and elutions therefrom) which place intermediate purity factor IX fractions for extensive periods of time in solutions having low concentrations of salts. No factor IX_a assays or evaluations of thrombogenicity are reported.

(D) Bajaj, S. P., et al. *Preparative Biochemistry* 11(4) 397–412 (1981) report a factor IX preparation derived from a technique involving four sequential separation procedures involving the use of barium citrate adsorption and elution, ammonium sulfate fractionation, DEAE-Sephadex® chromatography, and heparin-agarose chromatography. Crude and intermediate purity factor IX are maintained in low molarity salt solutions for considerable periods during and between each separation procedure. No enzymatic assays for factor IX_a or evaluations of thrombogenicity are reported.

(E) U.S. Pat. No. 4,786,726 discloses multi-step purification of factor IX involving the use of barium salt precipitation, anion exchange chromatography, ammonium sulfate precipitation, and dextran sulfate chromatography prior to further purification on an immunoaffinity column. The factor IX is manipulated with several extended periods of dialysis against factor IX-nonprotecting low molarity salt solutions.

(F) Goodall, A. H. et al. *Blood*, 59(3), 664–670 (1982), in a procedure representative of immunoaffinity methods, disclose the use of monoclonal antibodies to purify factor IX present in a prothrombin complex concentrate starting material, the concentrate having been prepared by standard methods, without regard to protecting the factor IX from activation or degradation in early stages.

Although monoclonal antibody columns have high specificity for factor IX and are effectively used to remove other contaminating coagulation factors, it is believed that there is not presently available an antibody affinity purification strategy which can bind factor IX and reject factor IX_a, (or vice-versa) (Smith, K. J. et al., *Thrombosis Research*, 33, 211–224, (1984)). In Tharakan, J. et al. *Vox. Sang.*, 58, 21–29 (1990), there is disclosed a high specific activity immunoaffinity purified factor IX product which is nonetheless contaminated by factor IX degradation products derived from traditionally prepared prothrombin complex starting material.

Expression of human clotting factor IX from recombinant DNA clones has been previously demonstrated in mammalian cells. Anson, D. S. et al., *Nature*, 315, 683–685 (1985), Jallat, S. et al., *EMBO Journal*, 9 (10), 3295–3301 (1990). Purification of such factor IX generally involves collection of a cell extract and then subjecting the extract to one or more of the above mentioned separation procedures. Protection of factor IX during purification from endogenous cell proteases may be accomplished by maximizing the amount of time factor IX-containing fractions are maintained in contact with relatively high concentrations of one or more of the organic or inorganic salts in accordance with the present invention.

It should be understood that a particular separation process or manipulation used in factor IX purification may be adversely affected by use of a relatively high salt concentration, as taught herein. If such is the case, the use of the relatively high salt concentration should, of course, be avoided for that particular separation process or manipulation, with the understanding that such high salt concentration can be used effectively in other purifying or accompanying steps which are not affected adversely, and in storage of the partially purified factor IX solution. Thus, one aspect of the present invention can be described as the recognition that protease-induced damage to factor IX which may occur within solutions containing low concentrations of salts may be substantially decreased by redesigning the purifying procedure to maximize the amount of time crude and intermediate purity factor IX fractions are maintained in a relatively high salt environment utilizing one or more soluble salts of the present invention.

A wide variety of different types of salts can be used in the practice of the present invention. One potential mechanism of action is that effective salts function by modifying the energetics of catalysis of relevant proteases. The effective salt may function by destabilizing proteases, by stabilizing the structure of factor IX, by reversibly altering the structure of factor IX so that it does not present to any particular protease those structural features which are normally attacked, by increasing the activation energy necessary to position and/or cleave factor IX at a protease active site, or by any combination of the above. Since the specific nature of all of the blood plasma or other proteases which can cleave factor IX is unknown, it is difficult to identify the specific mechanisms by which each of the useful proteolysis inhibitors functions. It is postulated that the factor IX molecule is strongly resistant to significant irreversible alterations In its tertiary structure caused by soluble inorganic or organic salts.

It is noted that there are many other causes for the denaturation of proteins (with resultant loss of catalytic activity or decay of three dimensional structure) during separation or manipulation procedures or during storage in solution. Examples of processes known to denature, break or otherwise adversely affect proteins in solution include shearing, binding to the surface of a vessel such as a glass wall, foaming of a solution, and oxidation of cysteine residues by atmospheric oxygen. The salt solutions useful in the practice of this invention may be effective also in arresting these other potential causes of factor IX degradation.

There can be used in the practice of this invention any water soluble inorganic or water soluble organic salt which is capable of reducing protease-caused cleavage of coagulation factor IX at a salt concentration which does not irreversibly alter the factor IX structure or cause factor IX to precipitate. The salts comprise a positively charged cationic component and a negatively charged anionic component which in aqueous media are dissociated to ionic form. The term "organic salt" refers to a salt in which either the cationic or anionic component thereof is a carbon-containing substance.

Examples of water soluble inorganic and organic salts for use as inhibitors of proteases which activate or degrade factor IX are: ammonium, alkali metal or alkaline earth metal halides; ammonium, alkali metal or alkaline earth metal thiocyanates; ammonium or alkali metal phosphates or sulfates; magnesium sulfate or phosphate; acetates of the alkaline earth or alkali metals; alkylammonium halides, including quaternary ammonium halides; and chlorides of imidazole, lysine and trihydroxyaminomethylmethane ("Tris").

Preferred salts for use in the practice of the present invention are sodium chloride; sodium and potassium thiocyanates; magnesium, potassium, and lithium chlorides; and sodium sulfate, with the most preferred salts being sodium chloride and sodium sulfate.

Effective concentrations of salts for use in the practice of the present invention can vary depending on the particular salt used. The lower limit of concentration is governed by how much of the salt is needed to inhibit factor IX degradation and activation. The upper limit is governed by that amount which can adversely affect factor IX and/or at which proportional benefits are not realized as the amount of salt is increased. Once the principle of protecting the factor IX molecule from degradation or proteolysis is recognized as taught herein, determination of optimal salt concentrations are readily obtained by simple incubation experiments as taught herein.

It should be appreciated also that there are factors which affect the range of concentrations over which a particular salt is effective. Such factors include the length of time or temperature for which protective effects are desired, the concentration of macromolecules, including proteases, present in the sample, and the nature of the manipulation, separation process and/or storage conditions under which protection is sought. These factors, coupled with the inherent ability of the particular salt to alter the energetics of proteolysis, should be taken into account respecting choice of effective concentrations.

It is believed that, for most applications, depending on the particular salt used, it will be satisfactory to use a salt concentration of about 0.4 to about 1.4 Molar.

It should be understood that a higher salt concentration can be used. Salts such as, for example, magnesium, potassium and lithium chloride and sodium sulfate are effective at the lower end of the amount range. These salts are particularly useful because factor IX-protecting effects can be realized at salt concentrations which are less likely to interfere with separation procedures such as those which involve the loading and binding of factor IX to an affinity column or ion exchange resin. Factor IX can therefore be protected continuously throughout a multistep purification procedure. There are other salts which will need to be used at the higher end of the range, for example, a concentration of at least about 0.7 Molar. A preferred amount range for the aforementioned preferred and most preferred salts is about 0.35 to about 3 Molar, dependent on the particular salt used.

As to relative effectiveness of various types of salts, in one series of tests 1 Molar concentrations of organic salts such as the hydrochlorides of Tris, lysine and imidazole, and sodium acetate were found to exhibit factor IX-stabilizing effects lower than those of a 1 M concentration of NaCl. Such organic salt solutions are protective, however, when compared to 0.15 M NaCl.

An important aspect of this invention is that a high salt-containing intermediate purity fraction of factor IX, which has been partially purified by ion exchange chromatography or another separation process, but not yet subject to immunoaffinity chromatography or other separation process, may be stored effectively for prolonged periods of time, for example, at least about 12 hours at 4° C. or at least about 3 months when stored frozen. In such storage, degradation or activation of factor IX is inhibited by virtue of the use with the factor IX of a relatively high concentration of salt as disclosed herein. This serves as a clinically important alternative to storing such fractions in the presence of toxic organic protease inhibitors which may be very difficult to remove from the final product. There are numerous processing advantages associated with being able to store intermediate purity fractions of factor IX during its purification.

Even factor IX products of the highest purity may contain traces of numerous proteases which will eventually degrade or activate factor IX, especially if the product, even at low temperature, is subject to long term storage in liquid form. In accordance with this invention and as an alternative to lyophilization or freezing, salts may be added to purified factor IX products to make long term storage in liquid form possible. For the purpose of providing a therapeutic form of the factor IX composition, the concentration of the salt in the composition should be reduced to a clinically acceptable level, for example, to about 0.15 M or lower. This can be accomplished by procedures such as dialysis, or by diafiltration prior to distributing the product to a clinical facility or a patient.

The purified, therapeutic form of factor IX preparations which result from the practice of the invention are expected not to trigger unwanted clinical consequences (such as myocardial infarction, thrombosis, and disseminated intravascular coagulation) which may otherwise result from administration of currently available factor IX products. The example section below provides data which validate the increased safety of factor IX prepared according to the process of the present invention.

There follows a description of a preferred overall factor IX purification for use in the practice of the present invention.

The preferred purification utilizes cryoprecipitation of blood plasma to remove proteins such as factor VIII, followed by anion exchange chromatography which takes advantage of the specific adsorbability of vitamin K-dependent clotting factors, followed by immunoaffinity chromatography using monoclonal antibodies, the last mentioned separation process having great specificity for factor IX compared with other protein species.

The preferred anion exchange resin for purifying factor IX is a highly hydrophilic bead formed gel of epichlorohydrin-crosslinked dextran to which diethylaminoethyl ether (DEAE) exchange groups are attached such as DEAE-Sephadex® A-50 available from Pharmacia, Uppsala, Sweden. The temperature is maintained below 15° C., and preferably below 4° C., during the Sephadex adsorption equilibration process. Although factor IX is bound to DEAE-Sephadex® A-50 more quickly and completely at 20° C. than at 4° C., it is recommended that the temperature be as low as possible to minimize protease activity on factor IX. Accordingly, adsorption of factor IX at 4° C. is preferred. Factor IX_a is also minimized as a contaminant in the final product by maintaining factor IX fractions (until such time as they have been separated from remaining contaminating proteases on a monoclonal antibody column) in a low temperature environment (such as 4° C. or below) in the relatively high molarity salt solutions of the present invention.

According to the preferred practice of this invention, proteolysis of factor IX is deterred in the anion exchange chromatography eluates by maintaining the protein in the presence of one or more suitable organic or inorganic salts at relatively high molarity (for example, about 1 Molar or more) until immediately prior to subjecting the factor IX enriched fractions to the further separation procedure of immunoaffinity chromatography using monoclonal antibodies which are used to separate factor IX from remaining coagulation factors and other proteins. In preferred form, prior to treatment with monoclonal antibodies, the molarity

of the solution is reduced to some extent, for example, to about 0.5 M. The affinity of the monoclonal antibodies for factor IX separates it from remaining materials which would tend to activate factor IX to factor IX_a and/or degrade factor IX to peptides of altered length and/or conformation. After separating factor IX from the monoclonal antibodies, it can be recovered, and even stored, in dilute form, if desired.

In connection with evaluating the purity of factor IX compositions that are capable of being produced by the present invention, two parameters, protein concentration and specific activity, must be determined.

Measurements which determine the protein concentrations in factor IX samples vary depending upon the precise method used. In the practice of this invention, the concentration of protein in factor IX samples is determined at 280 nm based on an extinction coefficient for a 10 mg/ml solution of pure factor IX of 13.7 units in a one centimeter pathway. Corrections for Rayleigh scattering are made following the methods of Bloom, J. W. et al., *Biochemistry*, 18, 4419–4425 (1979). See also Shapiro, S. S. et al., *Thromb. Diath. Haemorr.*, 16, 469 (1966).

Many assay methods have been reported in the art for evaluation of the specific activity (units factor IX activity/mg protein) of factor IX compositions. Such assay methods often fail to make corrections for contamination of the factor IX sample by factor IX_a. As a result, it is difficult to evaluate the purity and safety of factor IX therapeutics produced by different methods and/or assayed according to different protocols.

Some of the assays which are presently used include a two stage clotting assay, (Leibman, H. A. et al., *Proc. Natl. Acad. Sci.*, USA, 82, 3879–3883 (1985)); an assay based on the single stage activated partial thromboplastin time ("APTT"), Smith, K. J. et al., *Blood*, 72, 1269–1277 (1988); and assays which are modifications of the APTT test, for example, Jenny, R. et al., *Preparative Biochemistry*, 16, 227–245 (1986). Assays based on antigenic potency of factor IX have also been proposed as a measure of purity (Smith, K. J. et al., *Thromb. Haemostas.*, 58, 349 (1987)).

For the purpose of the present invention, the specific activity of factor IX compositions is assayed according to the single stage activated partial throinboplastin time "APTT" procedure of Smith, K. J. et al. *Blood*, 72, 1269–1277 (1988).

In prior art assays, many investigators have also used as a factor IX standard human plasma samples to which an assumed potency of 1.0 unit/ml is assigned. However, it must be emphasized that normal human plasma often contains a factor IX concentration significantly different from 1.0 unit/ml. For present purposes, reference is made to the International Reference Standard of factor IX, WHO #1, supplied by the World Health Organization.

Literature values for the specific activity of purified factor IX have been found to range from 130 to 220 units/mg. Differences in methods of protein determination, assay technique and calibration standards probably account for the reported differences. Compare Smith, K. J. et al., *Bloodp*, 72, 1269–1277 (1988) (134–155 units/mg); Bajaj, P. S. et al., *Preparative Biochemistry*, 11(4), 397–412 (1981) (180–220 units/mg); Osterud, B. et al., *J. Biol. Chem.*, 253(17), 5946–5951, (1978) (207 units/mg); Jenny, R. et al., *Preparative Biochemistry*, 16, 227–245 (1986) (132 units/mg).

Comparing results from different laboratory groups which utilize different methods to assay factor IX_a is also very difficult. This is particularly important since factor IX and factor IX_a can each interfere with assays designed to detect the other. An additional contribution associated with the development of this invention comprises the provision of a high sensitivity assay for factor IX_a based on the partial thromboplastin test ("PTT"). The assay herein reported is not affected by the presence of factor IX, and as demonstrated in the example section below, validates the utility of the factor IX stabilizing processes of the present invention.

Therapeutic factor IX-containing compositions useful in the treatment of Christmas disease need not be comprised of factor IX at the absolute limit of specific activity as long as remaining protein contaminants will not cause detectable adverse clinical. effects in humans when such compositions are administered in otherwise therapeutic doses. As demonstrated in the example section below, the purification procedure of Example 1 can be used to produce factor IX for therapeutic use with a specific activity of at least 194 units/mg protein with a factor IX_a contamination level of less than 0.02% (w/w). Factor IX compositions suitable for injection into a patient can be prepared, for example, by reconstitution with a pharmacologically acceptable diluent of a lyophilized sample comprising purified factor IX and stabilizing salts.

Among the techniques which can be used to show the purity of the resultant factor IX, and the minimization of contaminating factor IX_a or factor IX degradation peptides in the therapeutic compositions of this invention, are sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting of electrophoresed factor IX-containing samples, and direct enzymatic assays for factor IX and factor IX_a.

In the practice of this invention, and because of difficulties in correlating in vitro assays with in vivo thrombogenic events, the potential thrombogenicity of purified factor IX preparations is evaluated using (1) the Wessler Rabbit Stasis Assay, (2) SDS-PAGE and Western blots to determine the amount of peptide(s) having an approximate molecular weight of 54 kDa, and (3) a new modified form of the APTT assay to determine the Level of factor IXa or of other peptides causing IXa-like activity. It is believed that the best currently available indicator of safety of therapeutic factor IX preparations is provided when the sample has a low response in the rabbit stasis test, contains (by SDS-PAGE) less than 10% of 54 kDa material, and gives further indication of having a low factor IX_a content as measured in a modified (see Example 3 below) APTT test.

EXAMPLES

The following examples are illustrative of the practice of the invention.

Example 1

Purification Of Factor IX

This example demonstrates the purification of preparative quantities of therapeutically useful factor IX from blood plasma utilizing high concentrations of sodium chloride at particular steps to prevent proteolytic damage to factor IX.

A commercial manufacturing quantity of frozen plasma was allowed to thaw slowly at about 0° C. producing supernatant and precipitated fractions (see Pool, J. G. et al., *Nature*, 203, 312 (1964)). The supernatant fraction of the plasma which remains after cryoprecipitation contains factor IX and much of the original concentrations of factors II, VII and X. To the supernatant plasma fraction at about 4° C. were added DEAE-Sephadex^R A-50 anion exchange beads (about 1.5 g/liter of supernatant). The resulting suspension was stirred gently for one hour in the cold as factor IX bound to the resin beads. Approximately 96% weight percent of the factor IX originally present in the plasma was localized on

the resin beads. Other prothrombin complex proteins also bind. The resin beads with retained protein were collected by filtering and then washed with a volume of wash buffer,. precooled to about 4° C., which was at least equal to the volume of the cryoprecipitate-free supernatant. The wash buffer comprised a solution of 0.2 M NaCl, 0.01 M Na_3 citrate, pH 7.0, and 0.04 unit/ml sodium heparin. The ionic strength of the wash buffer was insufficient to cause significant dissociation of factor IX from Sephadex® beads. Eluting buffer (an aqueous solution of 2M NaCl with 10 mM sodium citrate, pH 7.0, and precooled to about 4° C.) was added and then gently stirred with the resin beads for 30 minutes. The resulting eluate was collected and held or processed further. Elution of the resin beads may be repeated and the eluates combined. Temperature control was maintained at between 2 and 8° C. for each of the steps of the DEAE-Sephadex® binding and elution procedure.

The elution of the factor IX-enriched fraction (prothrombin complex) from the DEAE-Sephadex^R resin with a solution of 2M NaCl buffered with 10 mM sodium citrate, results in a combined eluate fraction whose final NaCl molarity (because of the lower ionic strength of the void volume solution) is approximately 1.0 Molar.

Once clarified and sterile filtered, the combined eluate (the "stable factor IX-enriched fraction" of this invention) was stored frozen at or below −40° C. prior to subsequent processing. Storage in the frozen state at or below −40° C. for 4 weeks or less does not affect the usefulness of the factor IX-containing samples. As a result of the stabilizing influence of the salts useful in the practice of the invention, storage for commercially practicable periods of time results in intermediate purity factor IX preparations having less than 10% loss of potency and showing little or no activation or degradation. This would not be the case if storage were effected in a buffer containing a lower molarity of salt, such as 0.15 M NaCl, even at −40° C.

Alternatively, the stable factor IX-enriched fraction was stored at 4° C. for up to 12 hours prior to being further processed. This procedure is. in contrast to prior art methods which teach that the eluate should be dialyzed or diafiltered extensively against a buffer of low salt concentration prior to further purification or clinical use.

Immediately prior to applying the "stable Factor IX enriched fraction" to a monoclonal antibody affinity column, it is diluted 1:1 with water reducing the sodium chloride concentration of the preparation to 0.5 Molar. The factor IX-containing prothrombin complex solution is loaded on a monoclonal antibody column, washed extensively and eluted with 3 M sodium thiocyanate. Testing of the eluate for degraded factor IX or for factor IX_a shows them to be present at very low levels, allowing completion of the fractionation.

Preparation and utilization of monoclonal antibodies for immunoaffinity chromatography of the stable factor IX enriched fraction follow well established procedures. Purification of factor IX sufficiently pure for the production of monoclonal antibodies followed the procedure of Osterud, B. et al., *J. Biol. Chem.*, 253(17), 5946–5951 (1978). Spleens from mice previously injected with highly purified factor IX were removed and cells therefrom fused according to a standard procedure. Brown, J. P., et al., *J. Biol. Chem.*, 225, 4980–4983 (1980). Details of preparing and operating the immunoadsorbent system are as described in the specification "Immunoadsorbent, and Method of Recovering Vitamin-K Dependent Protein Therewith" EPO Serial No. 84-301162.8, published on Sep. 12, 1984 bearing number 0 118 256.

Factor IX was eluted from the monoclonal antibody column in a solution of 3 M sodium thiocyanate, 50 mM Tris.HCl, 10 mM EDTA, pH 8.0, and then diafiltered against 50 mM NaCl, 5 mM histidine, pH 7.0. The diafiltered factor IX solution was then subject to ultrafiltration against a YM100 membrane (Amicon Co. Danvers, Mass.) having a molecular weight cutoff for globular proteins of 100 kDa, thereby allowing factor IX to pass through the membrane with retention of viral particles. The factor IX-containing filtrate was collected, and then subject to filter concentration against a membrane suitable for retaining globular proteins with molecular weights greater than 10 kDa. The purified factor IX solution was then frozen and stored at −70° C. prior to final processing, thereby accommodating a commercial production schedule. The purified factor IX solution was finally subject to chromatography on a bead-formed agarose gel containing positively charged aminohexyl groups (AH Sepharose® 4B, Pharmacia, Uppsala, Sweden). The agarose gel chromatography accomplishes no actual further enhancement of purity of factor IX with respect to other coagulation factors, but serves to concentrate factor IX and remove trace levels of factor IX antibody which may have leaked from the monoclonal antibody column. Other commercially available gels can also be used.

After allowing factor IX to bind to the agarose gel beads, the gel was washed with 0.15 M NaCl, 10 mM histidine.HCl, 5 mM lysine.HCL, pH 7.0. Factor IX was eluted from the gel with a solution of 0.05 M $CaCl_2$, 0.15 M NaCl, 10 mM histidine.HCl, pH 7.0. The eluate was then diafiltered against 66 mM NaCl, 10 mM histidine.HCl, 3% (w/v) mannitol, pH 7.0, clarified, sterile filtered and stored at −40° C. or below. Alternatively the filtered solution may be freeze dried. The resulting material is the "improved factor IX final product" of the invention and has a specific activity which averages 180–200 units activity/mg protein.

Example 2

A Comparison with Prior Art Products

It is disclosed in the prior art that prothrombin complex concentrate, in approximately 1M–2M of NaCl, should be subject to lengthy manipulations such as dialysis, taking perhaps 24 hours, to reduce the salt concentration of the factor IX-containing isolate to the physiological range (about 0.15 Molar) for clinical use, or prior to further purification under low ionic strength conditions.

A comparison was made of the clarified, filtered prothrombin complex concentrate maintained in a 1.0 M NaCl solution containing also 10 mM sodium citrate, pH 7.0 ("the stable factor IX enriched fraction" of Example 1) with a prior art-type DEAE-Sephadex anion exchange chromatography eluate (a prothrombin complex concentrate) which was, according to standard practice, subject to dialysis or diafiltration to reduce its salt concentration to the physiological range. Both types of eluate fractions were then subject to further purification using monoclonal antibodies as described above resulting in an "improved factor IX final product", and a typical prior art final product.

Table 1 shows purity results for the final factor IX product of Example 1 and typical prior art process final product. As can be seen from the table, individual lots of "improved factor IX final product" derived from "stable factor IX-enriched fraction" show much less contamination by factor IX_a than final product derived from DEAE Sephadex anion exchange eluates which, consistent with the prior art, were subject to diafiltration into isotonic buffer solution for 3–4 hours with subsequent frozen storage prior to being applied to an immunoaffinity column. Factor IX was assayed by the "APTT" method according to Smith, K. J. et al.,

Blood, 72, 1269–1277 (1988). Factor IX_a was assayed by the partial thromboplastin test, "PTT", Varadi, K. et al., *Thromb. Haemos.*, 35, 576–585 (1976) as modified according to the protocol of Example 3 below. The decreased contamination with factor IX_a in the improved product is further validated by the longer clotting times, in seconds, in the factor IX_a assay as presented in Table 1.

TABLE 1

Comparative Assay Results of Factor IX Final Products

	Factor IX units/ml	Factor IX_a units/ml	Clotting Time in IX_a assay, Sec. (1:10 Dilution)	FIX_a/FIX Ratio as %	FIX/FIX_a Ratio
Prior Art Factor IX Final Product					
Lot 1	98.0	0.4875	63.1	0.4974	201
Lot 2	100.0	0.4375	62.6	0.4375	229
Lot 3	103.0	0.2350	64.0	0.2282	438
Lot 4	102.0	0.3500	52.5	0.3431	291
Lot 5	115.0	0.2625	68.0	0.2283	438
Improved Process Factor IX Final Product					
Lot 6	78.0	0.0286	146.2	0.0376	2,727
Lot 7	92.0	0.0278	151.1	0.0302	3,309
Lot 8	125.0	0.0247	160.3	0.0198	5,060
Lot 9	102.0	0.0088	225.0	0.0086	11,591
Lot 10	110.0	0.0423	134.3	0.0385	2,600

Anion exchange chromatography eluates and final products derived therefrom, when analyzed using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Weber, K. et al., *J. Biol. Chem.*, 244, 4406–4412, (1969) or as modified by Laemli, U. K. *Nature*, 227, 680–685 (1970), using an acrylamide concentration gradient of 4 to 12%, demonstrate (see FIG. 1) that prior art-type anion exchange chromatography eluate (prothrombin complex) and resultant final product are heavily contaminated with a band of apparent molecular weight of 54,000, characteristic of factor IX_a, (and certain factor IX degradation peptides), whereas this contaminant is much less apparent in the gel lanes corresponding to the stable factor IX-enriched fraction of the invention and final product derived therefrom. The lanes of the slab gel (FIG. 1) are loaded with approximately 14 μg of protein in the case of DEAE concentrates and approximately 9 μg for final products derived therefrom. Lane 1 of the Figure shows appropriate molecular weight markers. Lanes 2, 4 and 6 show individual lots of stable factor IX-enriched fraction derived from DEAE-Sephadex® chromatography according to the procedure of Example 1. Lanes 3, 5 and 7 comprise samples of the respective final products derived therefrom. Lane 8 comprises a sample of DEAE concentrate prepared by a non-optimized prior art procedure which does not use high concentrations of salt to protect factor IX. Lanes 9 and 10 present duplicate samples of final product derived therefrom.

Factor IX_a contamination of the final factor IX products and anion exchange chromatography eluates (both produced via the improved process, and according to the prior art) was also compared using the highly sensitive Western blot technique. Factor IX_a was detected by cross-reactivity of anti-factor IX antibody. In this procedure, the test samples were subjected to electrophoresis in a 4 to 12% acrylamide gradient gel in the presence of sodium dodecyl sulfate detergent. The proteins were then blotted and immobilized onto a nitrocellulose sheet. The pattern was visualized using rabbit anti-serum to human factor IX and horseradish peroxidase conjugate of goat anti-rabbit IgG. Color was finally developed using 4-chloro-1-naphthol and hydrogen peroxide. (See Pasternack et al., *Nature*, 322,740 (1986)). FIG. 2 demonstrates that the new process leads to a final product having only traces of factor IX_a even when the gels are overloaded, as compared with the typical result of the prior art. The gel lanes in FIG. 2 correspond exactly to the gel lanes in FIG. 1 and represent the Western blot of a duplicate of the SDS-PAGE gel shown in FIG. 1. The gel lanes are loaded with approximately 14 μg for samples comprising DEAE concentrates and 0.07 μg for lanes containing final products.

Example 3

An Improved Assay for Factor IX_a in Factor IX Preparations

In connection with the work on the present invention, an improved coagulation assay for factor IX_a, or for degraded 54 kDa factor IX-derived peptides or other proteins having factor IX_a-like activity, was developed and standardized to provide the art relating to blood purification with an improved analytical method. The principle of the new coagulant assay, which is highly sensitive, is based on the partial thromboplastin test ("PTT") and further demonstrates the lack of factor IX_a activity in factor IX purified in accordance with the present invention. The assay is useful in predicting in vivo thrombogenicity of factor IX preparations, especially when used in conjunction with the in vivo assays of Example 7.

The potency of factor IX_a or of peptides conferring factor IX_a-like activity is calculated directly based on clotting time in a one step assay. The phospholipid reagent used in the assay was derived from bovine brain and was used without activator. Thrombofax®, Ortho Diagnostic Systems, Raritan, N.J. Typical PTT procedure was modified by adding $BaSO_4$-adsorbed bovine plasma to factor IX-deficient human plasma in the assay protocol thereby increasing the supply of labile factors V and VIII.

This method is used for the measurement of activated factor IX_a in factor IX final products, as well as for monitoring ili-process samples. The results from a spike test with factor IX samples showed good linearity and reproducibility for the factor IX_a assay in the range of 0.0005 IX_a units/ml to 0.05 IX_a units/ml. Very small quantities of factor IX_a in factor IX preparations can therefore be measured.

The effect of purified factor IX on the factor IX_a assay was also studied by comparison of factor IX_a spiked with factor IX dilutions and factor IX spiked with factor IX_a dilutions. Factor IX does not interfere with the factor IX_a assay. The assay is readily adapted to monitoring other factor IX preparative procedures. The factor IX_a concentration data provided in Table 1 were calculated according to this procedure.

Only extremely low levels of factor IX_a were found in the final monoclonal antibody-purified factor IX product produced according to the procedure of Example 1. Example 4 to 8 of the invention further demonstrate the purities and stabilities of factor IX which can be achieved by using various types of salt environments in accordance with the present invention.

Example 4

Stabilization of Factor IX at Different NaCl Concentrations

This example demonstrates the effects of different sodium chloride concentrations on the stability of factor IX in intermediate purity preparations (DEAE Sephadex® eluate fractions) over a 5-day period of storage at 4° C.

Prothrombin complex (containing factor IX, significant quantities of factors II, VII, X and numerous other contami-

nating proteins) is eluted from the DEAE-Sephadex® resin using 2M NaCl in accord with the procedure of Example 1. Both a first and a second eluate from the resin are collected and separately stored at 4° C. Eluted fractions achieve different final NaCl molarities (0.96–1.4 M) as the buffer present in the void volume of the resin must be displaced.

The samples of the eluates which were generated for this example are:

(A) from the 1st column elution at 0.956 M NaCl

(B) a sample of material (A) diluted to 0.524 M NaCl

(C) a sample of material (A) subject to ultrafiltration and diafiltration as in the typical prior art process, reducing the NaCl concentration to 0.056 M.

(D) a portion of sample (C) is on completion of the filtration procedure then brought back to 0.481 M of NaCl for analysis over 5 days.

(E) from the second column elution at 1.4 M of NaCl.

(F) a sample of material (E) diluted to 0.532M NaCl.

Once prepared, these samples were stored at 4° C. and then examined at zero, one, two and five days time for factor IX and for factor IX_a. Factor IX was assayed in a single stage activated partial thromboplastin time "APTT" procedure according to the method of Smith, K. J. et al. *Blood*, 72, 1269–1277 (1988), and factor IX, levels were determined using the new factor IX_a assay presented in Example 3. Table 2 demonstrates that NaCl of 1.4 M or higher concentration results in maximal protection of factor IX against protease activity and minimizes production of factor IX_a under the conditions indicated.

TABLE 2

The Effect of Sodium Chloride Concentration on the Stability of DEAE-Sephadex Anion Exchange Chromatography Eluate preparations at 4° C.

	Final NaCl Molarity	F.IX (%) U/ML	$F.IX_a$ (%) U/ML	F.IX (%) U/ML	$F.IX_a$ (%) U/ML
		0 Days		1 Day	
Sample A	0.956	16.5(100)	0.0500(100)	17.0(103)	0.0500(100)
Sample B	0.524	9.1(100)		8.2(90)	0.0385
Sample C	0.056	14.0(100)	0.0125(100)	9.5(68)	0.0260(208)
Sample D	0.481	11.0(100)		8.6(78)	0.0339
Sample E	1.40	4.1(100)	0.0143(100)	4.4(107)	0.0159(100)
Sample F	0.532	1.35(100)		1.2(89)	0.0079
		2 Days		5 Day	
Sample A	0.956	16.5(100)	0.0408(82)	19.8(118)	0.0909(223)
Sample B	0.524	6.2(68)	0.0400	3.9(43)	0.0667
Sample C	0.056	6.5(46)	0.0297(238)	2.5(19)	0.3226(2581)
Sample D	0.481	6.2(56)	0.0238	2.7(25)	0.0385
Sample E	1.40	3.8(86)	0.0100(70)	5.0(131)	0.0294(294)
Sample F	0.532	0.88(65)	0.0044	0.32(24)	0.0093

From Table 2, it can be seen that use of the present invention has a profound effect on the stability of factor IX in prothrombin complex fractions. The results for 0 and 5 day timepoints were also confirmed in the Western blot assay system of Example 2 using an anti-factor IX monoclonal antibody which also reacts with factor IX_a and other 54 kDa factor IX-derived peptides.

Example 5

Effect Of Salt Concentration On Factor IX Stability at Two Different Temperatures

This example illustrates that the use of a relatively high concentration of salt in accordance with the present invention is effective over a wide temperature range.

A sample of DEAE Sephadex® eluate produced by the procedure of Example 1 above, which had been clarified and sterile filtered and containing 1M of NaCl and 10 mM sodium citrate, pH 7.0, was thawed after storage at −80° C. Separate five ml aliquots of the sample were dialyzed overnight at either 3° C. or 25° C., against 1.0, 0.75, 0.5, or 0.15 M sodium chloride solutions and then analyzed by Western blots and in factor IX and IXa activity assays. At low concentrations of dialysis salt, factor IX activation is substantial—even at 3° C. For example, when held at 3° C. and at 0.15 M of NaCl, approximately two thirds of the original factor IX is degraded after 12 hours. Factor IX_a levels were also observed to drop in samples dialyzed at low salt concentration, at 3 or 25° C., providing further evidence of the potential extent of proteolysis of prothrombin concentrates.

FIG. 3 presents the result of a Western blot at 25° C. Results at 3° C. were very similar. The respective lanes of the blot are (1) molecular weight markers; (2) a control consisting of frozen DEAE concentrate-eluate from DEAE-Sephadex® resin maintained in high salt according to the practice of this invention; (3) a thawed sample of DEAE concentrate (produced according to the procedure of Example 1 and containing also approximately 1M of NaCl) applied directly to the parent acrylamide gel without overnight dialysis; (4) a sample of DEAE concentrate produced according to the procedure of Example 1 and dialyzed overnight against 1.0 M NaCl; (5) a sample as in (4) above but dialyzed against 0.75 M NaCl; (6) a sample as in (4) above but dialyzed against 0.5 M NaCl; and (7) a sample as in (4) above but dialyzed against 0.15 M NaCl.

Example 6

Use Of Different Salts

This example demonstrates that a variety of soluble salts exhibit factor IX-protective effects.

A sample of DEAE Sephadex® eluate produced according to the procedure of Example 1 and containing 1 M of NaCl and 10 mM sodium citrate, pH 7.0 was clarified and sterile filtered prior to storage at −80° C. Separate five ml aliquots of the thawed solution were dialyzed overnight at 3°

C. against 1 Molar solutions of sodium acetate, lithium chloride, magnesium chloride, potassium chloride, sodium chloride or sodium sulfate. The dialyzed solutions were assayed for factor IX, factor IX_a and also analyzed in the non-activated partial thromboplastin time "NAPTT" test (Kingdon, H. S. et al., *Thromb. Diath. Haemorrh.*, 33, 617–631 (1975)). The results are summarized in Table 3 and were confirmed with Western blots. It can be seen that LiCl, KCl, NaCl, $MgCl_2$ and Na_2SO_4 are strongly factor-IX protecting at 1.0 14 concentration. It is noted that such effects can be demonstrated in both certain strongly salting in salts (Na or KSCN) and certain moderately salting out salts (KCl and Na_2SO_4).

The sodium acetate dialyzate showed considerably more factor IXa production than solutions of the other soluble salts although less factor IX activation and degradation were noted therein than would be expected using a 0.15M NaCl dialysis solution.

TABLE 3

Low Temperature Stability of Factor IX in 1M Concentrations of Various Salts

Sample		FIX	FIX_a	NAPTT	
No.	1M Salt	u/ml	u/ml	Dilution	%
1	Na Acetate	25.5	0.130	1:10	45
				1:100	82
2	LiCl	20.5	0.0294	1:10	62
				1:100	91
3	KCL	20.5	0.0200	1:10	90
				1:100	94
4	$MgCl_2$	19.0	0.0016	1:10	Did not clot
				1:100	106
5	Na_2SO_4	20.5	0.0192	1:10	100
				1:100	108
6	NaCl	21.0	0.0247	1:10	90
				1:100	99
Control	NaCl (thawed DEAE Sephadex eluate, without dialysis, after overnight storage.)	21.0	0.0333	1:7	77
				1:100	91

Example 7

Factor IX Purity As Validated By The Wessler Rabbit Stasis Assay.

This example demonstrates that factor IX purified in accord with the process of Example 1 does not cause unwanted coagulation as measured by the in vivo Wessler Rabbit Stasis Assay for thrombogenicity.

Factor IX preparations consisting of prior art DEAE Sephadex® eluates (diafiltered against physiologically isotonic buffer) and factor IX final products produced according to the practice of this invention (using anion exchange chromatography and held at high sodium chloride concentration prior to subsequent immunoaffinity chromatography) were injected in vivo into isolated, ligated sections of rabbit jugular veins according to the procedure of Wessler et al., *J. Appl. Physiol.* 14:943–946 (1959) to assess the formation of stasis thrombi.

Scoring was accomplished following the system of Wessler, et al. according to the size of the clot wherein a $^{+}4$ clot represents the largest size of clot which can normally be generated with thrombogenic materials in the size and type of vessel selected and $^{+}1$ is the smallest such clot which can be visibly detected. The results of the evaluations of the prior art compositions, and those of the present invention show that, in contrast to the use of compositions of the prior art, compositions of this invention show no adverse effects in vivo up to concentrations of factor IX on a per kg weight basis well beyond that which would be administered to a human hemophilia B patient. It is proposed that use of this assay in conjunction with analysis of SDS PAGE gels for 54 kDa peptides, and also the factor IXa assay (Example 3) is the best available method to predict of in vivo utility and safety of therapeutic factor IX preparations.

TABLE 4

Wessler Rabbit Stasis Assay for Thrombogenicity-Effect of Process Optimization on Thrombogenic Potential of Factor IX

Lot	Potency	Rabbit Scores								
		100 U/Kg			200 U/Kg			400 U/Kg		
Number	(u/vl)	1	2	3	1	2	3	1	2	3
Prior Art Process for Factor IX										
1	1060	$^{+}2$	$^{+}3$	$^{+}2$	$^{+}2$	$^{+}2$	$^{+}2$	$^{+}3$	$^{+}4$	$^{+}4$
2	500	$^{+}1$	$^{+}1$	$^{+}2$	$^{+}3$	$^{+}2$	$^{+}2$	$^{+}3$	$^{+}2$	$^{+}2$
3	230	$^{+}1$	$^{+}1$	$^{+}2$	$^{+}1$	$^{+}1$	$^{+}1$	$^{+}2$	$^{+}1$	$^{+}1$
4	255	0	0	0	$^{+}2$	$^{+}3$	$^{+}3$	$^{+}2$	$^{+}3$	$^{+}3$
5	1000	0	0	0	$^{+}1$	$^{+}2$	$^{+}1$	$^{+}2$	$^{+}1$	$^{+}2$
6	525	0	0	0	±	0	±	$^{+}1$	0	±
Optimized Process For Monoclonal Factor IX										
7	590	0	0	0				0	0	0
8	465	0	0	0				0	0	0
9	540	0	0	0				0	$^{+}1$	0
10	490	0	0	0				0	0	$^{+}1$
11	520	0	0	0				0	0	0

Example 8

Moderate Concentrations of Certain Salts are Effective to Protect Factor IX

This example demonstrates that certain soluble salts are effective in protecting factor IX when used at lower concentrations than are generally needed for sodium chloride. Since use of prothrombin complex concentrate can lead to dangerous clinical effects, recently developed purification strategies incorporate additional separation steps in their protocols to further purify factor IX. Not all of these separation techniques (for example, certain column technologies) can be made to work in high molarity salt conditions. For example, factor IX may not adhere to a particular monoclonal antibody affinity column if the salt concentration is much above 0.5 Molar. Therefore it would be desirable to identify soluble salts which exhibit factor IX-protective effects when present at moderate molarity such as between about 0.4 and about 0.7 Molar, or below.

Accordingly, separate 5 ml aliquots of a sample of DEAE concentrate, the "stable factor IX-enriched fraction" produced via the protocol of Example 1 (containing also 1.0 M NaCl buffered at pH 7.0 with 10 mM sodium citrate) and which had been stored frozen at ⁻80° C. were dialyzed overnight at 4° C. against 0.5 M solutions of NaCl, KCl, LiCl, Na_2SO_4 or $MgCl_2$. A citrate buffered 1.0 M NaCl dialyzate was used as a control. Assays for factor IX and factor IX_a were then performed, and the samples were screened (FIG. 4) in the Western blot system.

As is readily apparent from the blot in FIG. 4, buffered solutions of 0.5 M magnesium chloride (lane 3), sodium sulfate (lane 4), lithium chloride (lane 5) and potassium chloride (lane 6) each demonstrated factor IX stabilizing ability which is approximately equal to that of 1.0 M NaCl

(lane 8) and considerably better than that of 0.5 M NaCl (lane 7). Lane 9 in FIG. 4 shows appropriate molecular weight markers. Lane 1 shows a control of DEAE concentrate made by the typical prior art process and dialyzed against isotonic buffer prior Lo freezing-resulting in a high content of 54 kDa degraded peptide; and lane 2 shows a sample of frozen DEAE concentrate produced as eluate from DEAE-Sephadex® resin maintained in high salt according to the practice of this invention.

What is claimed is:

1. A method of treating Christmas disease in a patient which comprises administering to such patient an effective amount of a therapeutic composition comprising an aqueous solution of purified factor IX having a specific activity of greater than about 50 units factor IX activity/mg protein, wherein the composition is capable of being stored for a period of time of about 2 weeks at a storage temperature up to 15–30° C. and wherein at the end of said period of storage the composition has a factor IX specific activity of greater than about 50 units and is free of factor IX_a, degraded forms of factor IX and/or active forms of other clotting factors in concentrations which would tend to cause detectable adverse clinical effects in a human when said composition is administered in a therapeutic dose.

2. A method according to claim 1 wherein the therapeutic composition has a factor IX-specific activity of greater than about 100 units/mg protein before and after said period of storage.

3. A method according to claim 2 wherein the therapeutic composition has a factor IX-specific activity of greater than about 194 units/mg protein before and after said period of storage.

4. A method according to claim 1 wherein in the therapeutic composition said purified factor IX is prepared by separating contaminating proteins in solution therewith by a purification process comprising at least two sequential separation steps, said purification process including the steps of (a) adding to said solution of factor IX and other proteins one or more organic or inorganic salts to a concentration of from about 0.7 to about 3 M and (b) maintaining said salt concentration in said solution between purification steps until the factor IX has been separated from the contaminating proteins which tend to activate factor IX to factor IX_a and/or to degrade factor IX to peptides of altered length and/or conformation.

5. A method of treating Christmas disease in a patient which comprises administering to such patient an effective amount of a therapeutic composition comprising an aqueous solution of purified factor IX which is made using a method for purifying and preserving factor IX derived from human blood plasma or other source by separating factor IX from contaminating proteins which are in solution therewith by a purification process comprising at least two sequential separation steps, the improvement which comprises stabilizing factor IX in solution against activation to factor IX_a or against degradation to peptides of altered length and/or conformation by the steps of (a) adding one or more soluble organic or inorganic salts to the factor IX-containing solution to a concentration of from about 0.7 to about 3 M prior to or during a first separation step, which concentration is sufficient to prevent or substantially minimize activation of or degradation of factor IX, but which concentration is insufficient to cause precipitation of, irreversible alterations in, or denaturation of the factor IX molecule, and (b) maintaining the salt(s) in the solution at said concentration until at least through commencement of a subsequent separation step in which the factor IX is separated from those impurities which tend to activate factor IX to factor IX_a and/or to degrade factor IX to peptides of altered length and/or conformation.

6. A method of treating Christmas disease in a patient which comprises administering to such patient an effective amount of a therapeutic composition comprising an aqueous solution of purified fact-or IX which is made using a method of stabilizing factor IX which is derived from recombinant technology using bacterial, yeast or other cells against activation to factor IX_a or against degradation to peptides of altered length and/or conformation which method comprises adding one or more soluble organic or inorganic salts to a solution containing said factor IX to a total concentration of salt of from about 0.7 to about 3 M, which concentration is sufficient to prevent or substantially minimize catalytic action by proteases upon factor IX, but at a total concentration of said salt or salts insufficient to cause precipitation of, irreversible alterations in, or denaturation of the factor IX molecule.

7. A composition which comprises a lyophilized powder, solution, suspension, or frozen solution or suspension, of factor IX having a specific activity of factor IX above about 50 units/mg of protein and which includes, on a percent by weight of protein basis, no more than about 0.1% of factor IX_a, or no more than about 10% in total of one or more factor IX degradation peptides having molecular weights from approximately 40 to approximately 65 kDa.

8. The composition of claim 7 wherein said specific activity of factor IX is above about 194 units/mg of protein.

9. The composition of claim 7 wherein the composition is capable of being stored for a period of time of about 2 weeks at a storage temperature up to 15–30° C. and wherein at the end of said period of storage the composition has a factor IX specific activity of greater than about 50 units/mg of protein.

* * * * *

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Review Paper

Biology of factor IX

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Within the past 20 years or so, factor IX has been at the centre of particularly intensive studies of its physiology, pathology and biochemistry as well as its molecular genetics and biology. With the complete nucleotide sequence of its human gene determined in 1985 and the molecular defects of over 600 abnormal human factor IX genes analysed to date, factor IX is among the few mammalian proteins which have been exhaustively studied in almost every aspect. The enormous amount of information we now have on this medium-sized plasma protein sheds light on how a gene and its protein evolve, how the protein carries out a highly regulated, specific and pivotal role in the delicately balanced blood coagulation reaction, and the correlation between clinical presentations and its highly diverse molecular mechanism of defects. This wealth of knowledge makes factor IX an excellent model for deeper study, such as truly quantitative analysis of its structure–function relationship and *in vivo* function and regulation. It will also provide a sound foundation which may lead to improved treatment of haemophilia B and perhaps to its cure. This paper attempts to review the recent progress in research on factor IX.

Key words: Haemophilia, factor IX, molecular genetics, review.

Introduction

Blood coagulation is the principal mechanism which follows the initial platelet plug formation to stop blood loss after vascular injury.[1] The basic mechanism of blood coagulation and its regulation involves more than 20 protein factors in addition to calcium ion and phospholipids.[1,2] In this mechanism, factor IX plays a crucial role occupying a key juncture of the intrinsic pathway involving factor XI and the extrinsic pathway involving factor VII and tissue factor (Figure 1). After activation by these pathways, factor IX in turn activates factor X in the presence of factor VIII, Ca^{2+} and phospholipid surface. A deficiency of factor IX in the circulation results in a bleeding disorder, haemophilia B.

Recently, factor XI was shown to be activated by thrombin, resulting in significant revisions in the coagulation cascade (Figure 1).[3,4] These revisions have indicated the important roles of thrombin and factor XI in the initiation and maintenance of blood coagulation, and have provided an explanation for a lack of bleeding disorders due to deficiencies of factor XII, prekallikrein, and high molecular weight kininogen. Because both haemophilia A (deficiency of factor VIII) and haemophilia B patients bleed in spite of the normal amount of factor VII in their circulation and sufficient amount of tissue factor available, generation of factor Xa catalysed by the pathway involving factor IXa–factor VIII complex is obviously crucial for the stable maintenance of coagulation. Activation of factor IX and/or factor X by the factor VIIa–tissue factor complex upon vascular injury may be essential for the initiation of coagulation by generating the initial minute amount of thrombin which may, in turn, activate factor XI to factor XIa leading to subsequent production of factor IXa.[3] The activation pathway of factor X by factor VIIa–tissue factor, however, appears to be tightly controlled by lipoprotein-associated coagulation inhibitor (also called extrinsic pathway inhibitor), suggesting its transient role, if any, in maintenance of coagulation.[2,5,6] The activation pathway of factor IX by the factor VIIa–tissue factor complex, however, may still play an important role in maintenance of coagulation *in vivo*.[7]

This article reviews current knowledge of the biology of factor IX: its structure–function relationships, gene structure and abnormal genes, regulation of

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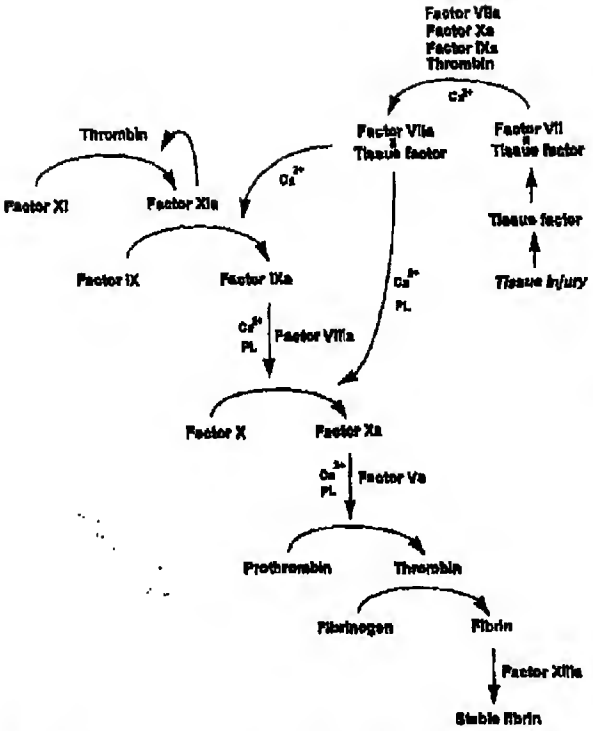

Figure 1. Basic mechanism of blood coagulation. PL indicates phospholipids. Activated forms of coagulation factors are shown with a suffix 'a'.

the gene and the current status of developing new therapy for haemophilia B.

Structure of factor IX

Human factor IX is synthesized as a prepro form of a single polypeptide chain (Figure 2).[8,9] Prepro factor IX is composed of several distinct domain (or module) structures. These include preleader (also called signal peptide) which spans amino acid (aa) −46 through −19, proleader (also called propeptide) spanning aa −18 through −1, Gla domain (the amino-terminal region of about 40 amino acid residues starting at aa +1) containing twelve γ-carboxylated glutamic acid (Gla) residues, a short hydrophobic sequence, two epidermal growth factor-like domains (each about 40 amino acid residues in length), a linking sequence, activation peptide (35 amino acid residues in length) and catalytic subunit of 235 amino acid residues. Intron positions relative to the amino acid sequences divide these domain structures in a characteristic manner. During secretion, both signal peptide and propeptide are cleaved off and the mature factor IX (plasma form of 415 amino acid residues in length) is produced. Although factor IX cDNA has three Met codons in the same reading frame clustered in the amino-terminal end region at aa −46, −41 and −39, the third Met residue at nucleotide (nt) −39 has a sequence (ATCATGG) which matches best with the Kozak consensus sequence (NNPuNNATGGNN),[10] suggesting that this Met could be the primary translation start site. This is further supported by the sequences of different species which have conserved Met at aa −39 but are missing a Met residue at −41 (dog, rat and mouse) and at −46 (dog and rat).[11] According to the ATG scanning model,[12] the first ATG at −46 may still be used for translation initiation albeit at low level. Prepro factor IX undergoes several co- and post-translational modifications. Its signal peptide is cleaved off by signal peptidase during the secretion of the nascent polypeptide chain, and the propeptide sequence is eventually cleaved off by a processing protease during the secretion of factor IX protein. Proteases which may be responsible for removing propeptides with a dibasic amino acid sequence at its carboxyl-terminus have been isolated and their cDNAs cloned.[13] These proteases are either metalloendopeptidase[13] or subtilisin type proteases (PACE).[14] In co-expression experiments with factor IX in Chinese hamster ovary (CHO) cells, PACE could enhance propeptide cleavage which takes place late in the secretory pathway.[15] Whether both or only one of these different endopeptidases are responsible for processing in the liver is not known.

The mature plasma factor IX is a single polypeptide chain starting with Tyr at aa +1 and ending with Thr at aa +415. Interestingly, all other homologous vitamin K dependent proteins of the coagulation system have Ala at this position (Figure 2).[9] Study by mutagenesis has shown that replacement of the Tyr residue at this position with Ala significantly improves the cleavage of the propeptide with little effect on γ-carboxylation of recombinant factor IX.[16] Why factor IX maintains a Tyr residue at this position is not known. It may have some biological significance in the overall regulation of coagulation. The plasma factor IX is secreted from hepatocytes into the bloodstream. During blood coagulation, the plasma factor IX undergoes limited proteolyses which free a 35 amino acid residue-long activation peptide, converting itself to the activated form, factor IXa. This is catalysed by either factor XIa in the presence of Ca^{2+} ions or factor VIIa in the presence of tissue factor and Ca^{2+} ions. Factor IXa is composed of a light chain (the amino-terminal half, aa 1–145) containing five structural domains with various functions to regulate the factor IX and a heavy chain (the carboxyl-terminal half, aa 180–415) containing the protease domain (catalytic subunit).

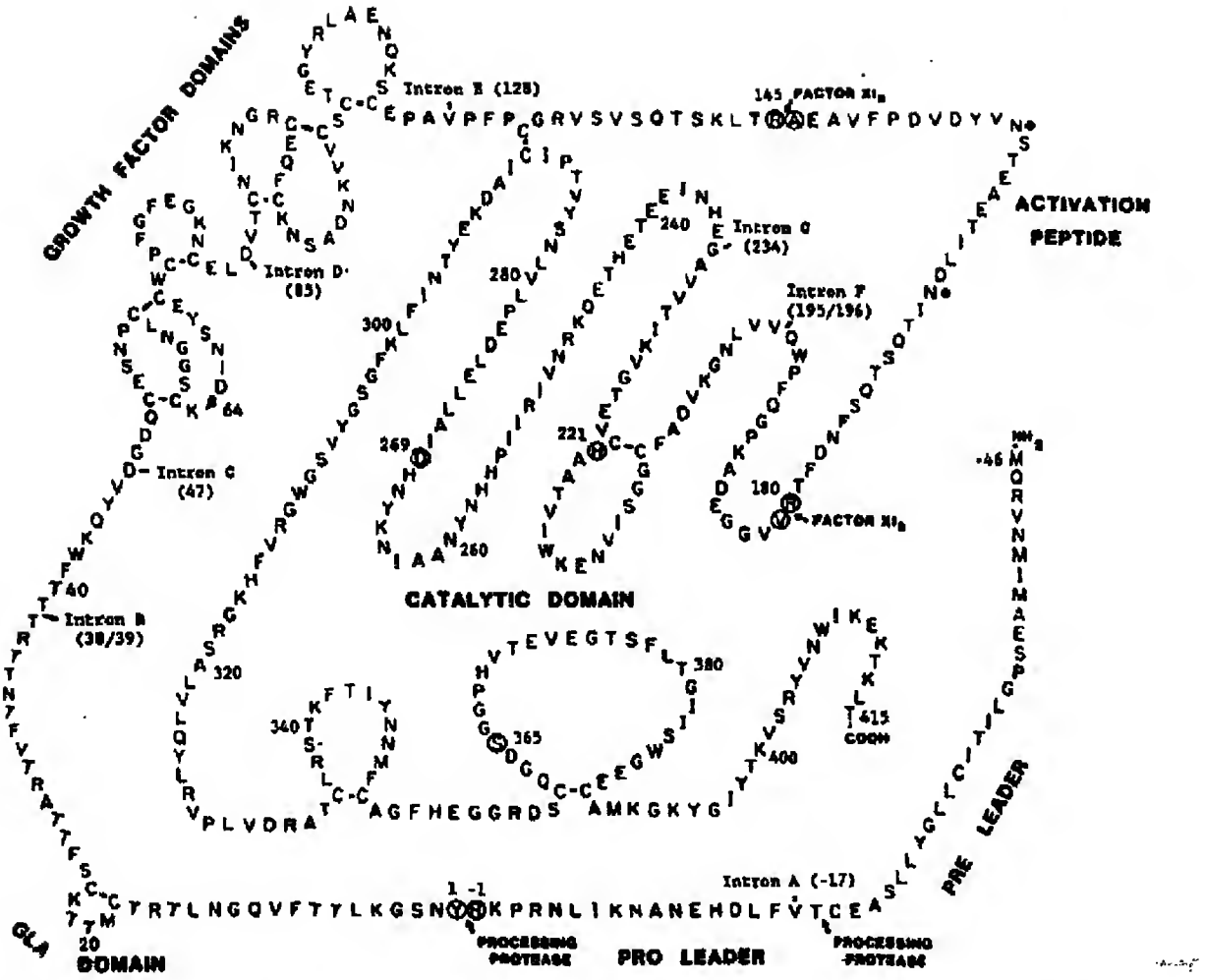

Figure 2. Amino acid sequence and tentative domain structure of human prepro factor IX. Numbering of amino acid sequence: positive numbers for the mature form of factor IX starting with Tyr at +1; negative numbers in reverse direction for preproleader sequence starting with Arg at −1. Intron positions in relation to the amino acid sequence are shown with residue numbers in parentheses. Arrows show the locations of peptide bonds which are cleaved during processing and activation of the prepro factor IX. Circled residues in the catalytic subunit indicate key amino acid residues involved in the active site. Modified from Yoshitake *et al.*[9]

The propeptide sequence of factor IX (aa −18 to −1) plays an important role in the vitamin K dependent γ-carboxylation of twelve glutamic acid residues contained in the Gla domain.[17,18] The importance of propeptide is also shown for other similar vitamin K dependent factors such as protein C.[19] Gla residues in the Gla domain play an essential role in the biological function of factor IX as Ca^{2+} binding sites. Studies on abnormal factor IX genes and a series of mutagenesis analyses carried out on the Gla domain and propeptide sequence have shed light on the mechanism responsible for the modification.[17,18] The propeptide alone without the Gla domain can serve as the recognition site for vitamin K dependent carboxylase which is embedded in the rough endoplasmic reticulum membrane. This is supported by the finding that synthetic propeptides alone can augment the carboxylase activity.[17,18] The maintenance of the approximate size of the intact propeptide is apparently important for its function, and some residues of the propeptide including those at aa −18, −17, −16 (Phe; conserved among vitamin K dependent proteins), −15, and −10 (Ala; conserved) are critical for the reaction. In these studies, Arg residues at aa −4 and −1 have been shown not to be critical

for the γ-carboxylation reaction. Interestingly, however, mutant factor IX molecules, factor $IX_{Cambridge}$ (Arg-1 changed to Ser)[20] and factor $IX_{San\ Dimas}$ (Arg-4 changed to Glu)[21] which are transported into the circulation with propeptides uncleaved, show decreased levels of γ-carboxylation. Mutations at aa −1 and −4 not only inhibit the proper cleavage of the peptide bond between Arg-1 and Tyr+1, but also may affect γ-carboxylation to some extent by changing the conformation of the propeptide and Gla domains. In γ-carboxylation, propeptide bound to carboxylase may function to anchor the nascent factor IX polypeptic chain so that the active site of carboxylase specifically recognizes the unmodified Gla domain region and scans the domain modifying the twelve Glu residues in the region to Gla residues.[17,18]

Recently, a cDNA clone for membrane-integrated, vitamin K dependent γ-glutamyl carboxylase was isolated.[22] When expressed in COS-1 cells and CHO cells, this carboxylase can augment the *in vitro* γ-carboxylase activity of microsomal preparations by 17- and 16-fold, respectively,[23] agreeing reasonably well with the previous observation.[22] In contrast, transient co-transfection of the γ-carboxylase expression vector into factor IX-expressing CHO cells did not improve the specific procoagulant activity of secreted factor IX, suggesting that the γ-carboxylation of factor IX is not limited by the expression of the vitamin K dependent γ-carboxylation alone.[23]

A very high expression of factor IX in hepatoma cells (>100 μg/ml medium) in culture results in a poor specific activity (only 1.5%) of factor IX, apparently due to poor γ-carboxylation.[24] When a relatively high level of recombinant factor IX (1–3 μg/10^6 cells/day) is produced by various heterologous cell lines such as BHK cells and CHO cells, its specific activity is also low, varying in a range of 25–70%.[25] Furthermore, a significant fraction (20%) of factor IX secreted from CHO cells escapes a proper cleavage of propeptide, resulting in inactive factor IX molecules with the propeptide still attached.[26] These data indicate that cultured cells such as BHK cells and CHO cells have mechanisms required for various co- and post-translational modifications with rather low, limited capacities.

Studies on the vitamin K dependent proteins have provided evidence of a specific biological role of propeptide in protein biosynthesis. The propeptide of von Willebrand factor has been shown to be required for multimerization of this protein, providing another function for such sequences.[27–29] Interestingly, the propeptide (except the first Thr residue) and Gla domain of factor IX are coded by a second exon, suggesting that these two adjacent unique domains are evolutionally one unit (Figure 2).[9] Several mutant factor IX genes containing mutations in the Gla domain,[30,31] such as factor $IX_{Chapel\ Hill}$[32] which has its Glu27 replaced with Val, provide invaluable information on the structures required for the function of the Gla domain. The Gla domain binds calcium ions with a moderately low binding affinity (average $K_d = 0.8\ \mu M$).[33,34] Binding of calcium ions to the Gla domain is required for its conformational rearrangement from a disordered form to an ordered and organized form involving the epidermal growth factor (EGF)-like domain. This conformational rearrangement is essential for factor IX to bind to negatively charged phospholipid vesicles provided *in vivo* by activated platelets resulting in its localization and augmentation of activation. Recently, the X-ray crystallographic structure of the Gla domain of prothrombin fragment 1 was determined.[35,36] This structure shows that in the absence of calcium ions, most of the Gla domain (aa 1–35) is substantially disordered. However, when the fragment 1 was crystallized in the presence of Ca^{2+}, the structure of the Gla domain was found to be well ordered, giving enough intensity of diffracted X-ray for a detailed analysis. This agrees well with the above observations obtained from experiments in solution. The Gla domain is composed of four separate short α-helices. The Gla domain of prothrombin fragment 1 binds seven Ca^{2+} ions containing four trapped between two parallel structures formed of two segments including residues 7 and 8, and residues 20, 21, 27 and 30. All Gla residues found in prothrombin are also conserved in factor IX, suggesting the similar Ca^{2+} binding may be expected for factor IX. A mutation (Gla27 replaced with Val) found in factor $IX_{Chapel\ Hill}$[32] therefore, apparently disturbs an important Ca^{2+} binding site in the Gla domain. Furthermore, mutagenesis analyses suggest that both Gla20 and Gla21 are required for maintenance of the structure recognized by factor XIa in activation, and that Gla21, but not Gla20, is also necessary for the calcium-dependent conformational change and endothelial binding of factor IX.[37] Factor IX, however, contains two more Gla residues (aa 36 and 40) which are not shared in prothrombin. Whether or not these are also involved in extra Ca^{2+} ion binding is not known. Binding of human factor IX to endothelial cells requires a small region of the Gla domain spanning residues 3–11.[38]

Two EGF domains in factor IX do not have any growth factor-like activity[39] and may have conformations of antiparallel pleated sheets as shown for factor X.[40] Only the first epidermal growth factor-like domain contains a high-affinity calcium binding site.[33,41] Binding calcium ion to this domain is essential to initiate conformational rearrangements involving the Gla domain.[33,34] The first EGF domain

(NH_2-terminal domain, corresponding to aa 47 through 84) undergoes at least three types of post-translational modifications. These include erythro-β-hydroxylation of Asp64,[42] O-glycosidically linked di- or trisaccharide (D-Xylpa1-3-D-Glcb1-O-Ser53, or one more D-Xyl extension) in human or bovine factor IX, respectively,[43] and three disulphide bond formations. β-Hydroxylation of Asp64 forming β-hydroxyaspartate (Hya), which is catalysed by a 2-oxoglutarate-dependent dioxygenase in liver microsomes, is only partial in factor IX (~30% complete). This is markedly different from other proteins such as factor X which undergoes a complete modification at this site. Dioxygenase does not require vitamin K for its activity, and β-hydroxylation is a reaction independent of γ-carboxylation.[44] By using inhibitors that block aspartyl β-hydroxylation of recombinant human factor IX, the Hya residue in factor IX was demonstrated to be non-essential for factor IX function as well as for binding to endothelial cells.

By a series of intrinsic protein fluorescent studies with various portions of factor IX, the first EGF domain was further studied for its high-affinity calcium binding site(s) (half saturation at ~40 μM Ca^{2+}).[33,34] This site is present independent of the state of carboxylation of the Gla domain. When Asp64 is not β-hydroxylated, the EGF domain still maintains a high-affinity Ca^{2+} binding site, although with K_d = 200–300 μM. Calcium ion binding at the high affinity site in the first EGF domain appears to induce significant conformational changes in factor IX that are detected by changes in intrinsic protein fluorescence, higher resistance to Lys endopeptidase and less accessibility to disulphide bonds by reducing agents. Binding of Ca^{2+} to the Gla domain, which has about ten-fold lower affinity for Ca^{2+} compared with the high affinity site in the first EGF domain, is required to complete the conformational rearrangement involving Gla and EGF domains. This rearrangement is necessary for factor IX to bind to the membrane surface. As shown for protein C,[45] the EGF domain may also affect the conformation and activity of the catalytic subunit of factor IX. In addition to the part of Gla domain sequence (residues 3–11 and 21),[38] the first EGF domain may be involved in the binding of factor IX to endothelial cells with a high affinity (K_d = ~2 μM).[33,34] The importance of the first EGF domain for factor IX function is supported by the detrimental effects of many mutations found in this domain,[30,31] such as factor $IX_{Alabama}$ containing Asp47 replaced with Gly (10% of the normal factor IX activity),[46] factor $IX_{New\ London}$ containing Gln50 replaced with Pro (< 1% activity),[47] and factor $IX_{Hollywood}$ containing Pro55 replaced with Ala (11% activity).[48] Interestingly, once activated, factor $IX_{New\ London}$ shows about 17% of normal activity which is comparable with other abnormal factor IX with mutations in the first EGF domain. The delayed activation of factor $IX_{New\ London}$ is in part responsible for its lowered activity. The replacement of Gln50 with Pro may also disrupt factor VIII binding, as observed for factor $IX_{Alabama}$ which shows a reduced effect of factor VIII on activation of factor X by factor IX.[46] Replacement of Pro55 with Ala in factor $IX_{Hollywood}$ was speculated to disrupt a β-turn structure required for the putative antiparallel β-sheet conformation of this domain.[48]

By swapping domains, Lin *et al.*[49] found that the first EGF domain of factor X can functionally replace that of factor IX, but the second (COOH-terminal side) EGF domain of factor X cannot assume the function of the counterpart of factor IX. These data together with those mentioned above suggest that the first EGF domain may function as a scaffold for holding the Gla region in Ca^{2+}-induced conformational rearrangements.[34] The second EGF domain of factor IX was also suggested to be involved in interaction with factor VIII.[49] This is further supported by a mutant protein which has a replacement of Asn92 with His in the second EGF domain.[50] Hertzberg *et al.*[51] reported that the second EGF domain and the protease domain of factor Xa in a chimera with the factor IX amino-terminal half including signal peptide, propeptide, Gla domain and the first EGF domain are sufficient to interact with factor Va. More recently, the first EGF domain was found to be required for factor IX activation by factor VIIa–tissue factor pathway, but not by the factor IXa pathway. It is also essential for optimal activation of factor X by factor IXa/factor VIIIa/phospholipid complex, but for neither phospholipid nor factor VIIIa binding to factor IXa (P. Bajaj, personal communication).

Most information on the structure–function relationship of the rest of the factor IX molecule, including the protease domain (catalytic subunit), has come from analysis of a large number of natural mutations, particularly missense mutations, which are distributed throughout the molecule.[30] The COOH-terminal side of the second EGF domain is connected to a linking region (aa 129–145). This short sequence is not clear except for Cys132 which is involved in a disulphide bond with Cys239 of the heavy chain, and Arg145 which is involved in one of the two proteolytic cleavage sites for activation of factor IX.[52] Mutant factor IX molecules with Cys132 replaced with Arg (factor IX_{Dakar})[30] and Arg145 replaced with Cys (factor $IX_{Cardiff}$ and others)[53] or His (factor $IX_{Chapel\ Hill}$ and others)[52] support the importance of these residues. During proteolytic activation, cleavages of two peptide

bonds, one between Arg145 and Ala146 and the other between Arg180 and Val181, release an activation peptide of 35 amino acid residues.[54] Structural requirements for the activation peptide appear not to be stringent except the immediate neighbouring sequences of the proteolytic cleavage sites which are involved in specific interactions with the activating enzyme. Absence of missense mutations, except the above-mentioned, in the regions of the linking and activation peptide, agrees with the notion that these regions function as spacers and are generally permissive for various amino acid sequence changes.

Mutations found in the catalytic subunit are also distributed throughout the domain. Mutations in some regions of the catalytic subunit, such as Pro287 replaced with Leu, Ala291 replaced with Pro, and Thr296 replaced with Met, apparently destabilize the protein or are detrimental for protein secretion, resulting in low-antigen, low-activity type variants.[30,31] Mutations in the highly conserved areas such as Gly363 to Val, Pro368 to Thr or Gly367 to Arg in the immediate neighbourhood of the active site residue (Ser365) cause a severe disorder, suggesting that these sequences are essential for keeping the active site structure functional. For example, factor $IX_{Eagle\ Rock}$ which has Val instead of Gly363, is apparently as stable as the normal factor IX, but cannot form a correct active site conformation because of the side chain of Val.[55]

Haemophilia Bm phenotype is characterized by its prolonged partial thromboplastin time and prothrombin time with ox (but not human) brain tissue factor,[56] and has mutations in the two distinct regions, an activation site area (Arg180–182) and another area (residues 390–397).[30,31] These mutant factor IX proteins likely interfere with the proper binding of factor VII (or VIIa) to the bovine tissue factor.

A large number of amino acid residues (about 60% of the factor IX sequence) apparently serve just as spacer sequences to maintain the overall factor IX protein structure, and are replaceable with most other amino acid residues with different side chains without resulting in haemophilia B.[57]

More recently, a high affinity Ca^{2+} binding site (K_d = ~500 μM) in the catalytic subunit was reported to be possibly involved in binding of factor IXa to factor VIII.[58] Carbohydrate chains attached to factor IX may play an important role in activation or function of factor IX as observed for factor X.[59]

Structure of factor IX gene

Complementary DNA and gene of human factor IX were cloned and their complete nucleotide sequences have been determined.[8,9,60] The nucleotide numbering originally employed for the complete contiguous sequence[9] has become the standard system for the gene and is used in this article. Factor IX is composed of eight exons in a span of about 34 kilobase (kb) pairs (Figure 3).[7] The size of the gene, however, may be as large as 40 kb depending on unidentified regulatory elements located in the 5′ and/or 3′ flanking sequences. The factor IX gene is located on the X-chromosome at q27 in an order of centromere—HPRT at q26—FIX at q27.1—fragile site at q27.3—Factor VIII site at q28—telomere.[61–63]

Bottema *et al.*[64] reported that the G + C content of the factor IX gene (40%) which is in general agreement with that of mammalian genomes cannot be explained by C to T or G to A transition alone at the CpG sites. The mutation rate at CpG sites is elevated about 24- and 7.7-fold relative to other transitions and transversions, respectively. Given the enhanced mutation rates at CpG, two-fold and three-fold mutational enhancement for transitions and transversions, respectively, at

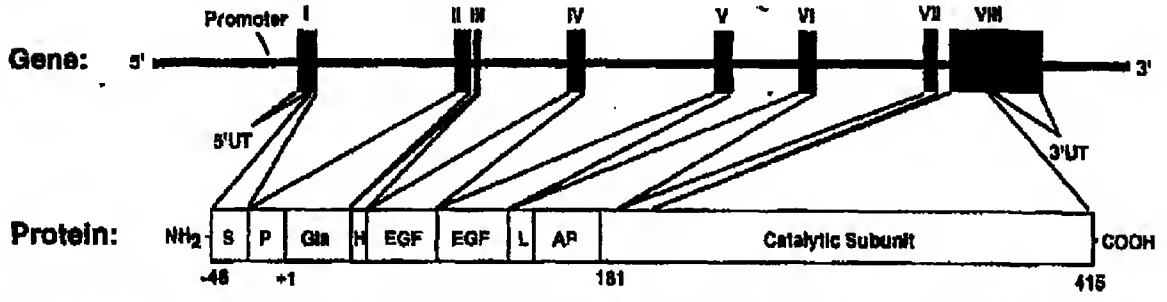

Figure 3. Organization of the human factor IX gene and domain structures of factor IX molecule. Exons are shown as solid vertical bars with exon numbers. S, P, Gla, H, EGF, L and AP represent signal peptide, propeptide, Gla domain, hydrophobic sequence, EGF-like domain, link sequence and activation peptide, respectively. Corresponding exons and domains are shown by lines. Numbers below domain structures indicate corresponding amino acid (aa) residue numbers.

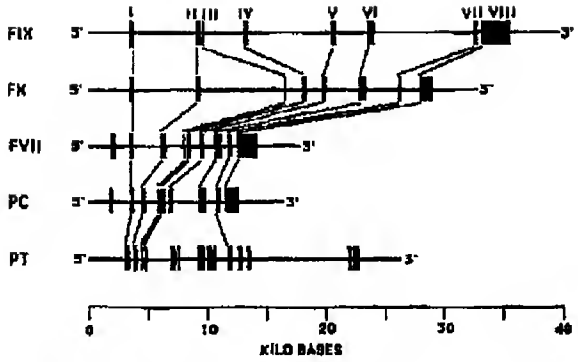

Figure 4. Comparison of gene organizations for factor IX (FIX), factor X (FX), factor VII (FVII), protein C (PC), and prothrombin (PT). Exons are depicted by solid vertical bars. Corresponding exons are shown by thin lines. Modified from Kurachi and Chen.[10]

non-CpG sites would be sufficient to produce the low G + C content of the factor IX gene. The G + C content in some other genes such as the protein C gene (over 50%) is significantly higher than that of the factor IX gene. Although the precise reason for this difference is not known, speculated reasonings may include that the specific location of each gene in chromatin which may vary in their susceptibility to base changes and/or the evolutional pressure to minimize the number of mutations in some crucial genes maintaining any functional changes at minimum.

The exon–intron organization including splicing phases of the factor IX gene are surprisingly similar to several other vitamin K dependent coagulation factors, indicating a typical divergent evolution involving a large number of point and minor mutations after exon shuffling events.[9] The overall sizes vary largely among these homologous genes due to different sizes of corresponding introns (Figure 4). Sizes of these genes range from 11 kb for the protein C gene[65] to 34 kb for the factor IX gene.[9] Protein S[66] and prothrombin[67] have only the first three exons homologous to factor IX. These exons encode its prepro leader sequence, Gla domain and a short hydrophobic sequence, but the rest of the molecules have grossly different structures. Prothrombin has two kringles and a protease domain with different exon–intron organization, while protein S has four EGF domains followed by a domain similar to steroid hormone binding protein. Protein Z has a similar organization to factor IX and other closely related factors, although it lacks two crucial amino acid residues required for the protease active site formation and does not have protease activity.[68,69] The number of introns and splicing phases in the catalytic domain of factor IX is identical to those of the genes for factor X,[70] factor VII[71] and protein C,[65] but distinctly different from those for prothrombin which has more introns in the domain.

Eight exons of the factor IX gene encode distinct domain structures (Figures 2 and 3). The 5′ end untranslated (UT) sequence and the signal peptide are encoded by the first exon. The entire propeptide (except the first Thr residue at −18 which is coded by the first exon) and Gla domain are encoded as one genetic unit by the second exon corresponding to their coordinated functions in the vitamin K dependent γ-carboxylation of glutamic acid residues in the Gla domain.[9] Other introns are also present at positions separating various unique domains.

Currently, more than ten polymorphic sites have been identified in or near the human factor IX gene (Table 1). Most of these are identified as changes in restriction sites including Bam HI site in the 5′ immediate flanking region (nucleotide sequence: C or T),[72] Hinf I/ Dde I site in a 50 bp AT-rich insert in the first intron,[73] Xmn I in the third intron (G or C),[73] Taq I site in the fourth intron (C or T),[74] and Mnl I site in the sixth intron (originally identified as Thr/Ala dimorphism at aa 148).[75] A highly polymorphic site (A > G) in Japanese population was found in the first intron.[76] An intragenic Bam HI polymorphic site located at −500 bp 5′ to the Xmn I polymorphic site in intron 3 in about 50% of the African American population.[77] Msp I polymorphism in strong disequilibrium with the Taq I polymorphism in the fourth intron was also reported.[78] Most intragenic polymorphic sites are in strong linkage disequilibrium, and these polymorphic alleles co-segregate in 70–80% of Caucasian factor IX genes. The Bam HI polymorphic site in the 5′ end region and Hha I polymorphism found at 8 kb downstream in the 3′ flanking sequence[77,80] are in equilibrium. Extragenic polymorphic Sst I site (detected with DXS99) and Taq I site (detected with DXS102) in the 5′ upstream flanking sequence and the factor IX gene may have a 3–5% chance of recombination due to cross-over events in meioses.[81,82] Linkage disequilibrium among the intragenic polymorphic sites and the possibility of recombination for the extragenic sites significantly hamper the usage of these for carrier detection and prenatal diagnosis. The overall usefulness of these polymorphisms in carrier detection is about 90% for whites and blacks. Frequencies of these polymorphisms vary largely among ethnic groups.[83] Except for the A/G polymorphic site in the first intron,[76] all intragenic restriction fragment-length polymorphisms which are present in Caucasians and African Americans are absent or extremely rare in Asians.[83]

Besides the polymorphic AT-rich sequence in the first intron, tandem purine and pyrimidine dinucleo-

Table 1. Allele frequencies of polymorphisms of human factor IX gene in various populations[a]

Restriction enzymes	Position	Allele (kb)	Caucasian		African American		Asian	
			No. of chromosomes	Frequency	No. of chromosomes	Frequency	No. of chromosomes	Frequency
Sst I	5′ extragenic	6	76	0.48	38	0.53	63	0.5
		9	81	0.52	34	0.47	63	0.5
Taq I	5′ extragenic	10	18	0.90				
		1.2	2	0.10				
Bam HI	5′ extragenic	25	90	0.94	50	0.64	100	1.0
		23	6	0.06	28	0.36	0	0.0
Hinf I/DdeI	intron 1	1.75	10	0.21	14	0.36	0	0.0
		1.70	36	0.79	26	0.64	30	1.0
Bam HI[b]	intron 3	15	32	1.0	11	0.52		
		13	0	0.0	10	0.48		
Xmn I	intron 3	11.5	42	0.7	12	1.0	76	1.0
		6.5	16	0.3	0	0.0	0	0.0
Taq I	intron 4	1.8	285	0.68	15	0.9	61	1.0
		1.4	129	0.32	2	0.1	0	0.0
Msp I	intron 4	2.4	40	0.8	22	0.4	57	1.0
		5.8	10	0.2	31	0.6	0	0.0
Mnl I[c]	exon 6	(Ala)	10	0.29	8	0.12	0	0.0
		(Thr)	25	0.71	60	0.88	95	1.0
Hha I	3′ extragenic	0.2	13	0.38	11	0.33	43	0.83
		0.15	21	0.62	22	0.67	9	0.17
purine/		I	4	0.29			0	0.00
pyrimidine		II	10	0.71			13	0.93
polymorphism[d]		III	0	0.00			1	0.07

[a] The data summarized in this table is a composite of data reported in papers including Hay *et al.*,[72] Winship *et al.*,[73] Camerino *et al.*,[74,75] Driscoll *et al.*,[77] Reiner *et al.*,[78] Mulligan *et al.*,[81] Hofker *et al.*,[82] Kurachi *et al.*,[30] Sarkar *et al.*[84] and personal communications (S.-H. Chen).

[b] This 2nd *Bam* HI site is located at 500 bp 5′ to the *Xmn* I polymorphic site and was detected by a *Bam* HI/*Sph* I digest.

[c] This is known as Thr/Ala-148 dimorphism which codes either for Thr or Ala.

[d] Allele I, $(GT)_4ATGC(GT)_4AG(AC)_4GCAT(AC)_3$; Allele II, $(GT)_4TGC(GT)_4AG(AC)_4GCAT(AC)_3$; Allele III, $(GT)_4ATGC(GT)_4AG(AC)_4GCAT(AC)_2$.

tide repeats which are polymorphic in most human races are also present in the 3′ UT sequence in the form of four different alleles.[84] These polymorphisms further improve carrier determination and prenatal diagnosis of haemophilia B.

The human factor IX gene contains many repetitive sequences such as Alu sequence and long interspersed element (Kpn I repetitive sequence, abbreviated as Line-1 or L-1). Five Alu sequences are present in introns and in the 3′ end immediate flanking region, while two Line-1 sequences, one partial element and one 6.1 kb complete element, are present in the fourth intron and in the 5′ flanking region, respectively.[9] Novel, short, interspersed repeat sequence (Ano) is also present in the first intron.[85]

Abnormal factor IX genes

To date, more than 600 haemophilia B patients have been studied for molecular defects in their factor IX genes.[30] Of 574 patient entries in the 1992 database (Third edition), 278 (48%) are unique and the rest are repeats which may be due to independent mutations or founder effects.

Mechanisms of mutation found in haemophilia B genes are highly heterogeneous, including at least 29 cases of complete or partial gene deletions or more complicated gene rearrangements, 50 short (less than 20 nucleotide) deletions or insertions or both, and a large number of single-base mutations which include 524 cases of missense, nonsense mutations, and mutations at splice sites as well as in the 5′ UTR and flanking sequence.[30,31] Gross and relatively large gene deletions, insertions and rearrangements, which can be rapidly detected by Southern blot analysis or polymerase chain reaction (PCR) as missing or rearranged DNA fragments, account for only about 4% of all mutations. Some of the gross gene deletions may be parts of much larger deletions which may span more than 500 kb in size.[31] All patients with gross gene deletions are severely affected. However, only two-thirds of those patients have developed alloantibodies

(inhibitors) against human factor IX infused during replacement therapy. Some patients with detectable factor IX antigens also develop alloantibody. These observations indicate that the development of inhibitors against the normal factor IX infused in the protein replacement therapy is primarily due to secondary factors such as treatment regimens and/or polymorphisms in the immune response system of individuals, but is not simply due to missing the entire factor IX antigen or to specific epitopes resulting from gross or partial gene deletions.

No obvious hot spots for deletion breaking points have been observed. However, factor $IX_{Seattle}$, which has about a 10 kb deletion spanning introns 4 through 6 has been shown to involve the 14 bp sequence (TAGAAGTTCACTT) duplicated 10 kb apart in introns 4 and 6.[86] In some cases, well-known repetitive sequences such as Alu sequences (highly abundant repetitive sequence of about 300 bp in length) are apparently involved.[87] An interesting mutant gene with an insertion is factor $IX_{El\ Salvador}$.[88] This has an insertion of about 6 kb extra sequence (which is likely an L-1 element) in the fourth intron within the 0.8 kb which spans between the 3′ end of exon 4 and the first Eco R1 site in this intron. Whether or not the insert sequence is directly responsible for this haemophilia B is yet to be determined. Line-1 element has a complete transcriptional unit with two open reading frames including retroviral reverse transcriptase-like sequence.[88] Line-1 insertion in exon 14 of the factor VIII gene has been reported as a novel mutational mechanism.[89] Line-1 sequence inserted in an intron may possibly generate an extra splicing set of sequences causing abnormal processing of the factor VIII gene in a mild haemophiliac.[90] Otherwise, Line-1 element insertion in introns may not be deleterious, and haemophilia in this type of kindred is incidental, probably due to an unidentified second mutation somewhere else in the gene.

About 40 mutant factor IX genes with small deletions such as one, two, three, four, seven and more than 13 bases in size have been found distributed in exons as well as in introns.[30,31] No specific hot spots for these small deletion mutations have been observed. Point mutations found in abnormal factor IX genes are distributed throughout the factor IX molecule, suggesting that the entire structure of the factor IX protein is highly optimized and that almost every part of the molecule is essential for maintaining its overall structure and/or specific function. A large part of the sequence is estimated to serve as spacers for maintaining the overall globular structure, as mentioned above.[37] This may be different from factor VIII with its dispensable, large central B domain. Currently, no function, other than its function as a large spacer (activation peptide), has been identified for the B domain of factor VIII.[91,92]

Missense mutations account for the majority (70%) of the point mutations in abnormal factor IX genes, while nonsense mutations account for about 16%.[30] These mutations, particularly missense mutations in factor IX genes, result in subtle changes of factor IX structure causing a wide spectrum of clinical severity and providing us with insights into the structure–function relationship of factor IX. Because of its relatively small size, availability of a large number of mutant genes with missense mutations and the complete gene structure, factor IX may be the most amenable protein among all coagulation factors for the exhaustive study of structure–function relationships.

Mechanisms responsible for the point mutations found in the factor IX gene are highly heterogeneous. Among them, CpG dinucleotide sequence has been clearly recognized as a mutational hot spot.[30,31] Eighteen (or 36 for the double strands of DNA) CpG sequences are present in the coding region, which is only a quarter of the possible random dinucleotide sequences. Endogenous methylase converts some of deoxycytidine of the CpG sequences to 5-methyldeoxycytidine which is then spontaneously converted to deoxythymidine by deamination. Because no cellular repair mechanisms are present for this conversion, the rate of mutation at CpG in factor IX genes is elevated at least 24-fold and 7.7-fold for transition and transversion, respectively, over the other random mutations at non-CpG sequences.[44,30,31] Interestingly, the lack of repair mechanism for this alone cannot account for the reduced frequency of CpG sequences in the gene.[44] About 45% of all unique point mutations found in factor IX genes are due to mutations at CpG sites. Some of these mutations observed may be due to founder effects. Data obtained on factor IX genes agree well with those for other genes such as the factor VIII gene.[93] Twelve CpG sites in the factor IX gene have base mutations once or multiple times. Mutations at CpG sites such as Thr296 replaced with Met are duplicated in unrelated families, further supporting these CpG sites as hot spots.[56] Some CpG sites do not have any mutations reported. The underlying mechanism for this is not known. It is possible, however, that these CpG sites are somehow inaccessible to methylase in the chromatin structure. Multiple different mutations at the same nucleotide sequences of CpG or at non-CpG sites have also occurred.[30,31] Some examples include replacements of nt −6G (5′ UTR) by A or C, nt +13A (5′ UTR) by G or C, or deletion, nt 6 365G in a codon for Arg-4 by T or A, nt 6 704T (splice site) by G or C, nt 10419G in a codon for Cys56 by C or A,

20524G in a codon for Val182 by C or T, nt 20566G (splice site) by T or A, nt 30992G in a codon for Ala291 by C or A and nt 31290C in a codon for Ala390 by T or A. These data further support the concept that the mechanisms responsible for the mutations are highly heterogeneous, and demonstrate that the factor IX structure has been extensively tested and refined by mutational events in the process of evolution.

An interesting case of somatic mosaicism of abnormal factor IX with a mutation (Cys350 to Ser due to G to C change) has been reported.[97] In a family affected with haemophilia B, a male member was very mildly affected (35% factor IX activity with 45% antigen level of normal). In this haemophilia B kindred, the two female members (daughter and granddaughter of the affected male) were moderately affected (3% activity and 4% antigen of the normal factor IX level). Factor X and factor VII levels as well as prothrombin time were normal. Among somatic tissues of the affected male analysed, about 10% of the total cells of both leucocytes and liver have the normal gene while about 90% of the cells have the mutant gene. However, the cells of kidney and smooth muscle have both normal and mutant genes about equally. These results indicate a somatic mosaicism, probably due to a replication or post-replication repair error during the first mitotic divisions in the zygote preceding implantation, or a half-chromatid mutation generated during meiosis that was not corrected before fertilization. In the leucocytes of the two female patients, both normal and mutant genes are present in an equal amount. These data suggest a possibility that not only liver but also leucocytes are of endoderm origin, which is contrary to the commonly held mesodermal origin for leucocytes. A rare case of severe haemophilia B in a girl due to non-random inactivation of a normal factor IX gene has also been reported.[98]

A class of abnormal genes which have mutations in the Leyden-specific region (LS-region, arbitrarily defined as a region from about nt −40 to +20) belong to the haemophilia B-Leyden phenotype, which shows a unique delay of the factor IX expression until the onset of puberty. Eighteen mutations have been found in the 5′ end region of the factor IX gene. Among these, one is located at nt −793 in the 5′ upstream, and the rest (17 mutations found) account for twelve unique mutations in the LS-region. Eleven of these mutations result in the Leyden phenotype.

Regulation of the factor IX gene expression

The factor IX gene is expressed in liver with a high tissue specificity.[99] Illegitimate expression in other tissues is also observed.[100]

During most of the gestational period, the factor IX

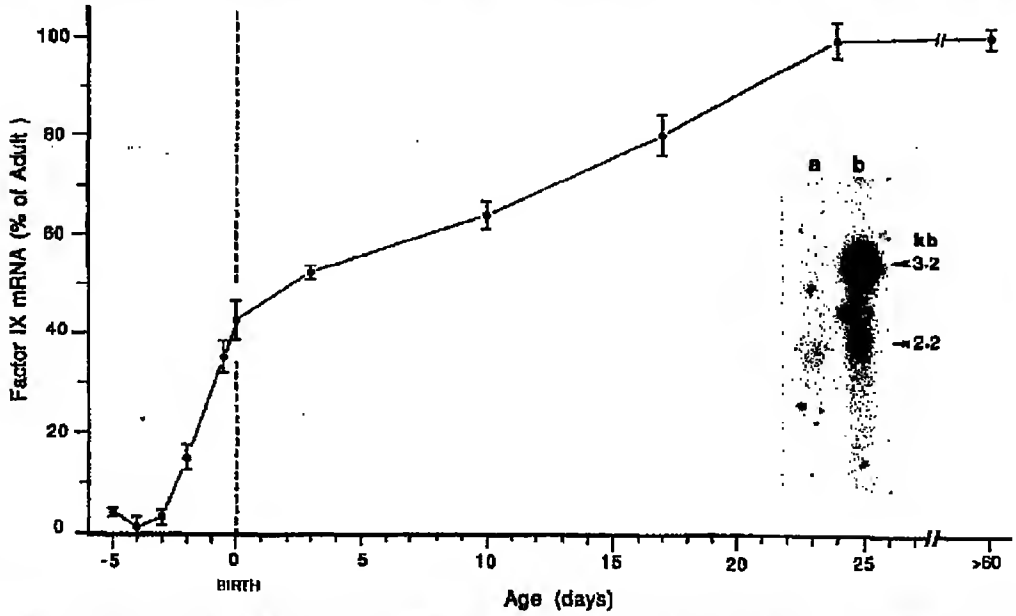

Figure 5. Steady-state liver mRNA levels for mouse factor IX at various developmental stages. Factor IX mRNA levels (solid dots) are shown as percentages of that of the adult. Vertical bars with short horizontal bars indicate standard errors (n: 6–8). The inset picture shows the results of Northern blot analysis of poly(A)$^+$RNA prepared from NIH/3T3 cells (lane 1) and mouse liver (lane 2). The numbers on the right indicate the sizes of the estimated transcripts. Taken from Yao *et al.*[100] with permission.

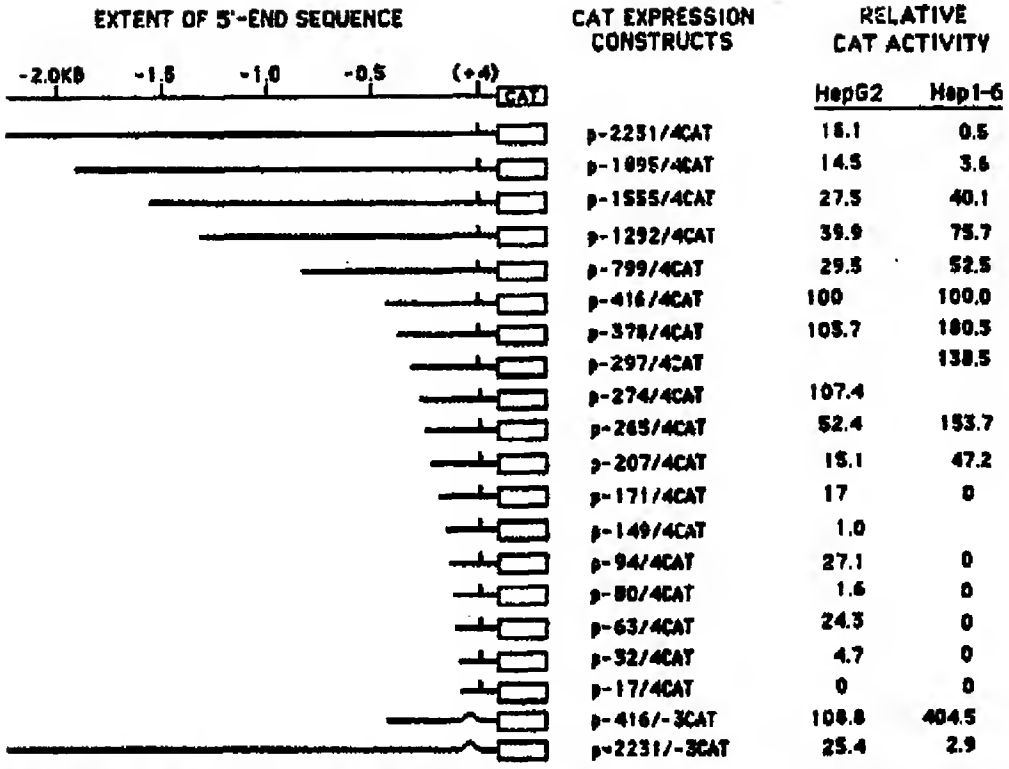

Figure 6. Analysis of the 5' end flanking sequence of the factor IX gene for promoter activity. Expression activities of CAT constructs containing various portions of the 5' end flanking sequence were assayed in HepG2 cells and Hep1-6 cells.[99] The 5' end sequence of the factor IX gene is shown with solid lines with the CAT gene shown as an open box at the 3' end. CAT activities relative to that of p-416/4CAT are shown in percentages. The thin lines connected in the middle indicate deleted areas. The 5' and 3' ends of the factor IX gene sequence contained in each CAT construct are shown by two numbers separated by a slash in the labellings.[9] p-416/29CAT, which contains a factor IX sequence extended to nt +29 at the 3' end, shows identical expression activity to that of p-416/4CAT. Deletions of the 3' end sequence from nt +29 up to −2 in p-416/29CAT did not effect expression activities of CAT constructs. However, further deletions beyond nt −3 position dramatically reduced the activity.

gene is expressed only at a low level (3–5% of the adult level) until the late stage of the third trimester. This was shown for humans, with limited data,[101] or lamb[102] and more completely for mice.[103] The developmental time curve of expression of the factor IX gene in mouse liver shows an induction of a high-level expression of the factor IX gene on day 18 of gestation (late stage of the third trimester) (Figure 5). The increased expression of the gene continues through birth followed by a rather gradual increase until reaching the adult level at weaning (20–24 days of age). At birth, the factor IX mRNA level is only 43% of the adult level, and the plasma factor IX activity level agrees well with the mRNA level. These results agree well with the limited data available on humans.[104,105] The low level of factor IX mRNA at birth may be responsible in part for haemorrhagic disorders in pre-term or term neonates. This condition, however, may be aggravated by generally poor vitamin K synthesis in neonates and, furthermore, if antibiotics or vitamin K analogues such as warfarin are given to the mother during the prenatal stage.[106] Other pathological conditions such as diarrhoea and cystic fibrosis also lower the vitamin K level, resulting in secondary haemorrhagic diseases.[107]

As in any other gene, the 5' end region of the factor IX gene contains various short sequences which function as *cis*-acting elements in its regulation.[99] Systematic analyses of these elements in the 5' end region have been carried out with expression vector constructs containing variously deleted 5' end region of the factor IX gene ligated to chloramphenicol acetyltransferase (CAT) gene as a reporter (Figure 6).[99] The schematic drawing of the overall organization of the major functional elements identified is shown in Figure 7. The

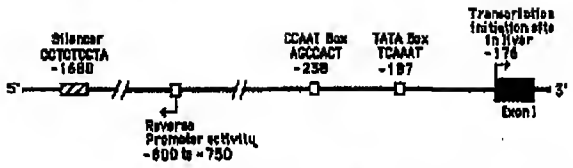

Figure 7. Locations of major functional elements in the 5′ end region of the factor IX gene. Nucleotide numbering system is from Yoshitake *et al.*[9] The nucleotide sequence of the silencer is of the complementary strand.

fundamental elements necessary for a high-level expression of the factor IX gene are contained in approximately the first 300 bp sequences of the 5′ end region (Figure 7). As more 5′ upstream sequences beyond nt −400 region are included into the CAT constructs, lower expression activities are observed (Figure 6). The sequence including up to about nt −1900 shows only a low-level activity (16% of the construct with a sequence up to nt −416). Even lower expression (less than 3%) is observed when the 5′ upstream sequence up to −6.9 kb is included in the expression vector. The factor IX gene does not have a typical TATA sequence in the 5′ end flanking sequence. However, according to the functional analysis data, sequences TCAAAT starting at nt −187 and AGCCACT starting at nt −238 have been tentatively identified as functional TATA box and CCAAT box, respectively. AGCCACT agrees well with the consensus CCAAT sequence.[109]

The locations of fundamental transcriptional elements of the factor IX gene agree well with the transcriptional start site placed in the region of nt −150 for CAT constructs[99] which was revised from the previously assigned site (+1 site).[109,110] The primary transcription start site in liver was determined to be at nt −176 in the 5′ upstream by primer extension and DNase I protection analyses with high-quality poly (A)⁺RNA preparations of human livers.[111,112] Reverse transcription-mediated PCR with primers at or downstream of nt −176, but not with one at nt −300 region where no signals for transcription initiation were observed, can amplify products, further supporting the

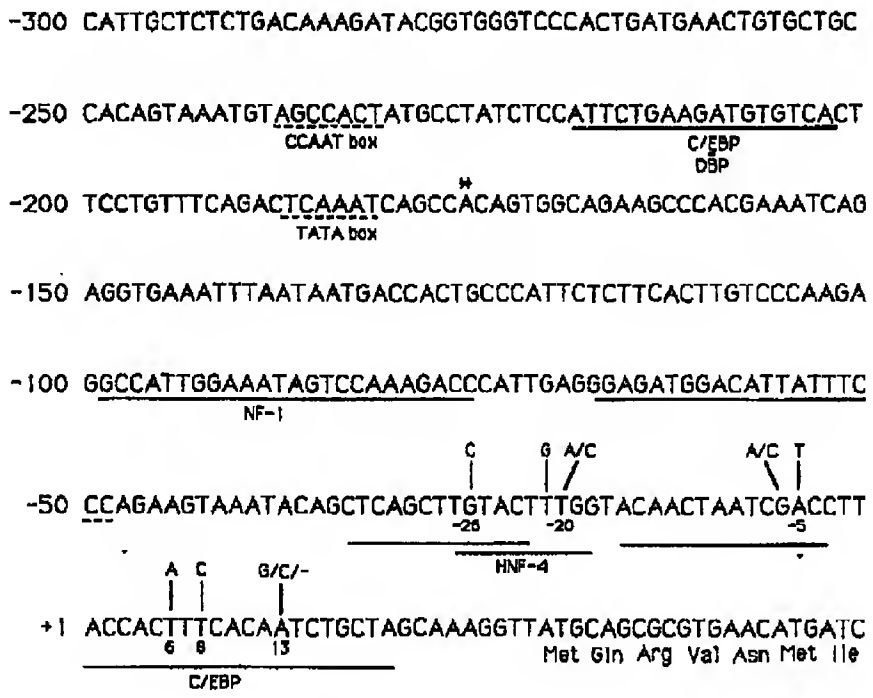

Figure 8. The nucleotide sequence of the 5′ end region (300 bp) with various elements. The numbers on the left indicate the old nucleotide numbering.[9] The revised primary transcription start site in liver is shown with an asterisk. Part of the signal peptide starting with the first Met residue at aa −46 is shown at the bottom line. Solid underlines indicate protein binding regions. Dotted underlines indicate tentatively identified functional CCAAT and TATA boxes. The LS-region is arbitrarily defined as the region roughly spanning nt −40 to +20. Mutations found in haemophilia $B_{-Leyden}$ genes at nt −20, −6, −5, +6, +8 and +13 are shown with short vertical bars with mutant sequences above the LS-region. The mutation at nt −26 (G to C change) does not show a Leyden phenotype.

initiation site in liver. A dog factor IX cDNA which has the 5′ extension at least up to −179 is also in good agreement with the revised 5′ upstream start site for the human gene.[113] The previously observed transcription initiation site (+1) is likely a secondary start site in liver or an artifact, probably due to poor quality of the RNA preparations used. The site, which is located in the middle of a region designated as LS-region, could be in a unique secondary structure which makes factor IX mRNA highly susceptible to degradation at this site. Crossley and Brownlee[114,115] reported functional analyses of the 5′ end promoter region for transcriptional activity using a CAT construct containing a 5′ end sequence up to nt −189 as a control for the full CAT activity. This construct, which contains the factor IX sequence up to nt −189, however, has only about 15% of the optimal constructs which contain a 5′ sequence up to nt −300.[99]

Structural elements homologous to the known liver-specific enhancers are also present in the 5′ end promoter region. These include TGGACC (partial LF-A1 or HNF-4 element) at nt −359 in sense strand and CTTTGGACT (PRI element) at nt −79 in antisense strand, which are also present in other genes such as α_1-antitrypsin, transferrin and antithrombin III genes.[99] The region, nt −76 through −99, containing CTTTGGACT has been reported to bind NF-1[116] which is originally identified as a liver-specific enhancer protein.[116,117]

Several negative regulatory elements (silencers) which are identical or similar to those found in other genes are present in the region of about nt −700 through −2000. These elements are responsible for the activity reduction observed for CAT constructs which contain various portions of sequence in this region.[99] Among them, a sequence spanning −1.4 kb to −1.7 kb contains two sequence elements, ATCCTCTCC starting at nt −1680 and CAATGGTT at nt −1621, which are similar to the well-characterized consensus silencer elements (negative regulatory elements).[99,118] When a sequence containing these elements was placed downstream of a CAT gene at the Bam HI site in p-416/+4CAT which contains the factor IX promoter sequence (nt +4 through −416), both orientations of this sequence (sense and reverse orientation) reduced the expression activity to 21–26% of p-416/+4CAT.[99] These results indicate that silencer elements in the region are actually functional in the factor IX gene. More silencer-like elements found in the 5′ end region include ACCTATGGAA starting at nt −726, CTGAATGGCT at nt −793 and CAATGACT at nt −1467. Interestingly, a very strong promoter activity in a reverse direction is present in a region spanning nt −700 to −750.[99] The sequence elements responsible for this reverse direction promoter are currently not known. No retroviral LTR-like sequences are present in this region. The presence of the reverse promoter region appears to coincide with a significant reduction (60–70%) of the normal expression activity of the factor IX gene (Figure 6).[99]

Important information on the regulation of the factor IX gene has also been obtained from transgenic mice experiments. Jallat *et al.*[119] have recently constructed transgenic mice carrying factor IX minigenes with the 5 kb sequence of the 5′ end immediate flanking region containing the promoter elements in addition to all the silencer elements detected in the *in vitro* assay and variously shortened intron sequences. Their results have clearly indicated that the liver-specific high expression of the factor IX gene can be achieved by various constructs with the 5 kb 5′ end flanking sequence as the promoter and the partial sequence of the first intron. A factor IX cDNA construct (containing no intron sequences) with the same 5 kb sequence of the 5′ end flanking region shows only a background level expression in transgenic mice. These observations strongly suggest that at least one intron as a set of splicing sequences or a putative enhancer element(s) which may be present in the first intron must be responsible for obviating the silencer activity in the 5′ end upstream sequence.

The data obtained from both *in vitro* and *in vivo* experiments indicate several important points, including: (i) high-level expression of the factor IX gene can be achieved by the elements contained within the sequence up to about nt −300, (ii) this expression activity is efficiently suppressed by multiple silencers present in the 5′ upstream region, and (iii) the reduced activity may be restored to a high level *in vivo* in the presence of the first intron partial sequence. The obviation of the silencer activity in the 5′ upstream sequence by the first intron sequence, therefore, appears to be a key mechanism underlying the overall regulation of the factor IX gene.

Important observations regarding the developmental regulation of the factor IX gene have been obtained from a unique class of haemophilia B, haemophilia B-Leyden.[120–122] While the normal factor IX gene is induced for its high-level expression at the perinatal stage,[103] Leyden phenotype factor IX genes are not expressed or not induced for their high expression until the onset of puberty.[120] Eleven unique single-base mutations so far found in haemophilia B-Leyden families include nt −21(T to G), −20(T to A or C), −6(G to A or C), −5(A to T), +6(T to A), +8(T to C) and +13(A to G or C or deletion).[30,121–129] Without any exceptions, all these mutations are contained in the LS-region (roughly from nt −40 to +20) in the 5′ untrans-

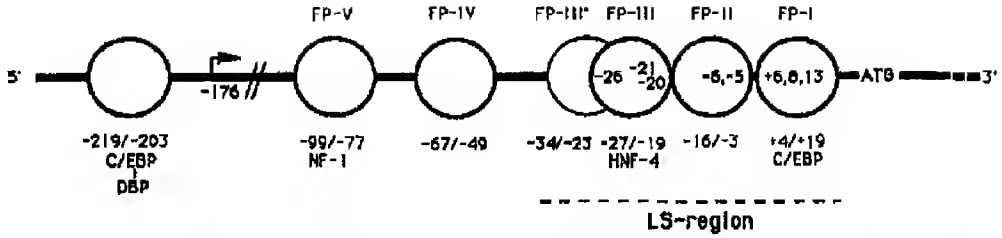

Figure 9. Protein binding at the LS-region and its neighbouring region. The human factor IX sequence is shown by a thick solid line. Proteins which bind to the region as evidenced by footprinting analyses are shown by circles. A circle with thin line indicates the protein binding in the factor IX-Leyden gene with a mutation at nt −20. Numbers in circles indicate the nucleotide residue locations of the natural mutations found in the Leyden phenotype factor IX genes. Numbers below circles indicate actual sequence regions of footprints. Known proteins are also shown below the numbers. An arrow indicates the primary transcription site in liver.

lated sequence of the factor IX gene (Figure 8). With the revised primary transcription initiation site of the factor IX gene,[99,111] the LS-region is located within the 5′ untranslated sequence. When mutations found at nt −20(T to A), −6(G to A) and +13(A to G or deletion) were tested for their effects using CAT expression vectors, all mutant sequences substantially reduced the expression activity of these constructs to 15–30% levels of the normal sequence,[112,122] indicating the importance of these sequences for the factor IX activity. As found by DNase I footprinting analyses, the normal LS-region and its neighbouring region bind five proteins[111,130] (schematically shown in Figure 9). These include three apparently new proteins, shown as footprints FP-II, FP-III′ and FP-IV,[111,130] in addition to recently reported HNF-1, C/EBP and HNF-4.[114,115,129] In the 5′ side neighbouring region, NF-1 and an unidentified new protein bind at a region spanning nt −99 to −76, and at a region spanning nt −67 to −44 (FP-IV), respectively.[130] Neither one of these new proteins is glucocorticoid receptor, nor androgen receptor.[99,111] Binding of C/EBP, which is present in most differentiated cells at significant levels, to a region spanning nt +3 to +19, is easily detected by both DNase 1 footprinting analysis and electrophoretic mobility shift analysis.[111,114,130] Recently, the protein binding to −20 region (FP-III, nt −17 to −27) was determined to be HNF-4, a protein of the steroid receptor superfamily.[115,129] HNF-4 binding to this region of the normal factor IX gene prohibits binding of another protein which binds to an overlapped androgen responsive element-like sequence (FP-III′, nt −36 to −22).[111,130] This protein, however, can competitively bind to the region of the factor IX gene if the gene has a mutation at nt −20 which causes a gross decrease in the binding affinity of HNF-4 to the region. This mutation-dependent competition between the two overlapped regions for protein binding was shown by both gel mobility shift assay and DNase I footprinting analysis.[111,130] The importance of HNF-4 binding to the region and the unidentified protein which binds to FP-III′ has been further supported by a drastic decrease in the expression activity of the factor IX gene with a mutation at nt −26.[115,129] Although Crossley *et al.*[115] reported that the protein which binds to the FP-III′ region is androgen receptor (AR), careful protein binding analyses have shown that it is not androgen receptor, but an ubiquitous protein present in nuclear extracts of liver as well as cultured cells which are not only androgen receptor-positive, such as T47D, LNCaP and HepG2, but also androgen receptor-negative cells such as CV1 and COS cells.[111,130] Glucocorticoid receptor does not bind to this region in good agreement with the results from the expression assay. When the Leyden phenotype mutations at nt +13, −6 or −20 are present in oligonucleotide sequences (double-stranded form) used in the electrophoretic mobility shift assay, the binding affinity of these proteins to the oligonucleotides is grossly decreased, agreeing well with reduced expression activities observed for the mutant sequences.[111,130] Interestingly, the 3′ half of the LS-region, where C/EBP binds at +13 subregion (FP-I), and the 5′ half, where two proteins (HNF-4 and an unidentified protein) bind in the region containing −20 and −6 subregions (FP-III and FP-II), apparently function with little cooperation.[122] The 3′ half, for instance, where C/EBP binds, may require a second unidentified element in the 5′ upstream which is not included in the CAT construct used in the assay. In this regard, Picketts *et al.*[131] recently reported that DBP interaction with C/EBP which binds at nt −202 to −219 region may synergistically confer its enhancer activity on a factor IX-Leyden gene and is responsible for the amelioration of haemophilia B-Leyden with a mutation at nt −5. DBP is induced for its expression in adulthood, but

not in childhood. This is an attractive mechanism to explain, at least in part, the Leyden phenotype. However, it has difficulties in explaining some important aspects of the Leyden phenotype. These include: (1) If DBP binding at the 5′ upstream can override the defects in the LS-region, why does DBP not ameliorate the mutation at nt −26, which is in the LS-region, after puberty? (2) Why does the normal factor IX gene which has the normal LS-region not significantly elevate its expression level after puberty as DBP increases its level? Whether or not DBP can selectively interact with any proteins, including C/EBP which binds at the 3′ half of the LS-region, is not known.

The LS-region of the transcript of the factor IX gene may assume some secondary structures such as stem loop structures, albeit not extensive, as predicted from the sequence.[122] The functional significance of stem loop structures in the untranslated region of transcripts has been well documented for other genes such as Tar element in HIV-1 or iron responsive elements in ferritin and transferrin genes.[132–134] A possible involvement of these unique structures of the LS-region in its function remains to be determined.

Development of alternative therapies for haemophilia B

Currently, haemophilia B is treated by plasma protein replacement therapy.[135] This therapy is effective, but exposes patients to possible risks of serious side-effects and complications such as contracting blood-borne viruses including hepatitis and HIV-1 viruses, thrombosis due to other coagulation factor contaminations, and inhibitor (alloantibody) development.[136] A large number of haemophilia patients (70–90%) who have received repeated plasma protein replacement therapy are already infected with HIV-1 viruses. In addition, frequent transfusions of factor IX preparation required in the therapy for severely affected patients are highly costly and significantly impair the quality of life of patients.

Large-scale production of recombinant human factor IX for safer protein replacement therapy by cultured mammalian cells is currently hampered by complicated post-translational modifications, such as γ-carboxylation, required for normal factor IX function.[25] Poor efficiency of such modification by cultured cells has been a serious problem in producing in quantity recombinant factor IX with a high specific activity. Unexpectedly, coexpression of cloned γ-carboxylase and factor IX cDNA did not improve γ-carboxylation of recombinant factor IX.[23] Information obtained from these studies, however, should eventually help to prepare recombinant mammalian cells which can express fully carboxylated factor IX. Such cells may be successfully used to produce much safer recombinant factor IX in quantity to substitute the plasma factor IX preparations currently in use.

A novel approach for an alternative haemophilia therapy includes somatic gene therapy.[137,138] This approach requires an *ex vivo* or *in vivo* transfer of the normal human factor IX gene (factor IX minigene constructed with the cDNA are widely used) into a target tissue of a patient, such as liver where the factor IX gene is normally expressed, or other tissues which can support long-term production of biologically active recombinant factor IX without any deleterious effects. If such an approach is developed, it may be able to obviate several serious side-effects of the current plasma replacement therapy.

Several cell types including rodent and haemophilic dog skin fibroblasts,[139–141] endothelial cells,[142] liver hepatocytes,[143] skeletal muscle cells,[144] and keratinocytes[145] have been tested for their ability to produce biologically active recombinant factor IX in culture. Reported recombinant factor IX preparations produced in these approaches have varied in their specific activities (~70–100% of the plasma factor IX). The variations are, in part, due to artifacts of the methods used to quantitate the recombinant factor IX secreted into medium. This problem, however, was recently solved by introduction of a simple pretreating procedure using serum with barium sulphate.[146]

When genetically modified skin fibroblasts were implanted *in dermis* or subcutaneously in mice or rats, recombinant factor IX was transiently expressed. Palmer *et al.*[140] reported that by using recombinant retroviruses containing cytomegalovirus promoter or retroviral long terminal repeat promoter, the recombinant human factor IX was produced at very high levels (~3.4 or 1.6 μg/10^6 cells/day in normal human diploid fibroblasts or in normal rat diploid fibroblasts) in culture. When these genetically modified cells were implanted into nude mice or rats, transient systemic levels of recombinant factor IX reached 0.18 μg and 0.022 μg/ml plasma, respectively. St Louis and Verma[139] originally reported systemic delivery of recombinant factor IX at a transient level of ~0.1 μg/ml serum in mice by implanting genetically modified mouse skin fibroblasts embedded in collagen under epidermis. A very inefficient systemic delivery of the produced recombinant factor IX (2–6%) as well as promoter inactivation and poor stability of the promoter used, were observed. These problems obscured the advantage of using skin fibroblasts for this purpose. Scharfmann *et al.*,[147] however, reported that use of housekeeping gene promoters such as dihydrophorate reductase may overcome some of

these problems. Recently, human applications of skin fibroblast gene therapy have been reported from China.[148] The MoMLV retroviral expression vector with its LTR as the promoter was used in these applications. One of the two mildly affected patients who received the therapy has shown a limited, transient improvement over several months. This approach still needs a substantial amount of systematic testing for its efficacy and safety before being applied to humans in this country.

Fully active recombinant human factor IX can be produced by rat capillary endothelial cells in culture at a level of 0.84 $\mu g/10^6$ cells/day.[149] A brief account of factor IX production by bovine adrenocortical endothelial cells is also reported.[141] These results indicate that endothelial cells have all the basic properties necessary to serve as a drug delivery vehicle for producing recombinant factor IX.

Skeletal myoblasts as an efficient gene transfer vehicle to obtain a high-level production of recombinant factor IX in the systemic circulation (~1 μg/ml in C3H mice) have been described.[140,145] Several important findings in this series of work include: (1) skeletal muscle cells can efficiently express foreign genes including factor IX at a high level; (2) skeletal muscle cells have mechanisms for post-translational modification producing human factor IX with a very high specific activity (81–90%); (3) efficiency of the systemic delivery of recombinant factor IX by muscles is surprisingly high (≥ 29%); (4) long-term expression *in vivo* can be achieved; (5) intramuscular implanted myoblasts can, not only fuse to host myofibres, but also survive as quiescent muscle precursor cells (muscle stem cells, presumably as satellite cells), further supporting the rationale to utilize myoblast-mediated gene transfer for developing a long-term stable gene therapy for haemophilia B. Extensive efforts targeting liver are in progress in multiple laboratories. Expression of human factor IX (0.071 $\mu g/10^6$ cells/day) by rabbit hepatocytes using retroviral vector containing cytomegalovirus promoter has been reported.[143] Ponder *et al.*[150] recently reported that implantation of bacterial β-galactosidase gene-tagged hepatocytes obtained from transgenic mice (C57BL/6) by intrasplenic injection resulted in deposition and long-term survival (> 6 months) of the transplanted cells in parenchyma, which amounts to 0.5% of the entire liver. A high-level systemic delivery of human α_1 antitrypsin (~5 μg/ml plasma) in a similar approach was also observed,[151] strongly suggesting that this approach may be feasible for developing somatic gene therapy for haemophilia B. The direct factor IX gene transfer into rat liver by receptor-mediated gene transfer has shown a transient expression of biologically active factor IX into circulation.[152] The short-lived expression of factor IX observed, however, must be much improved for developing a clinically acceptable gene therapy protocol for haemophilia B.

More recently, *in vivo* expression of factor IX by taking a route of *ex vivo* gene transfer with retrovirally transduced keratinocytes was reported.[145] The expression level was extremely low (≤ 1–2 ng/day/10^6 cells) and lasted less than a week, suggesting a need for more improvement before this procedure can serve the purpose. Low level expressions of dog factor IX (~ 6 ng/ml plasma) was also observed in partially hepatectomized haemophilia B dogs after infusion of factor retroviral vector.[153]

Currently, none of the approaches under investigation is ready for clinical testing for haemophilia in the United States. Within the next 1–3 years one or more of these approaches may be highly optimized for efficacy and safety, and become feasible for clinical applications.

Conclusion

To date, over 600 abnormal factor IX genes have been studied for their molecular mechanisms. This extensive study of factor IX in recent years is largely due to its clinical importance, the availability of its complete, contiguous nucleotide sequence which was determined in 1985, and development of readily usable technologies such as polymerase chain reactions. Furthermore, its multidomain structure with an amenable size for various protein chemical and recombinant DNA manipulations has made factor IX an exciting model for studying structure–function relationships of complex proteins.

With the enormous amount of data accumulated, new important directions of research on factor IX in the future appear to be regarding its *in vivo* role in the regulation of thrombosis and haemostasis, alternative therapy development including gene therapy and recombinant factor IX production for safer protein replacement therapy, and its regulation at the gene expression level. As one of the key factors in the blood coagulation cascade, factor IX will continue to serve as an invaluable model to provide fascinating insights into the intricate mechanism of blood coagulation and its regulation.

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References

1. Saito H. Normal haemostatic mechanisms. In: Ratnoff OD and Forbes CO, eds. *Disorders of Haemostasis*, 2nd ed. Philadelphia: WB Saunders, 1991: 18–47.
2. Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 1991; 30: 10363–10370.
3. Naito K, Fujikawa K. Activation of human blood coagulation factor XI independence of factor XII. *J Biol Chem* 1991; 266: 7353–7358.
4. Gailani D, Broze Jr GJ. Factor XI activation in a revised model of blood coagulation. *Science* 1991; 253: 909–912.
5. Broze Jr GJ, Girard TJ, Novotny WF. Regulation of coagulation by a multivalent Kunitz-type inhibitor. *Biochemistry* 1990; 29: 7539–7546.
6. Rapaport SI. The extrinsic pathway inhibitor: a regulation of tissue factor-dependent blood coagulation. *Thromb Haemostas* 1991; 66: 6–15.
7. Bauer KA, Kass BL, ten Cate H, *et al.* Factor IX is activated *in vivo* by the tissue factor mechanism. *Blood* 1990; 76: 731–736.
8. Kurachi K, Davie EW. Isolation and characterization of a cDNA coding for human factor IX. *Proc Natl Acad Sci USA* 1982; 79: 6461–6465.
9. Yoshitake S, Schach BG, Foster DC, *et al.* Nucleotide sequence of the gene for human factor IX (antihaemophilic factor B). *Biochemistry* 1985; 24: 3736–3750.
10. Kozak M. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J Biol Chem* 1991; 266: 19867–19870.
11. Pang CP, Crossley M, Brownlee GG. Comparative sequence analysis of mammalian factor IX promoters. *Nucleic Acids Res* 1990; 18: 6731–6732.
12. Bergenhem NCH, Venta PJ, Hopkins PJ, *et al.* Mutation creates an open reading frame within the 5' untranslated region of macaque erythrocyte carbonic anhydrase (CA) I mRNA that suppresses CA I expression and supports the scanning model for translation. *Proc Natl Acad Sci USA* 1992; 89: 8798–8802.
13. Kawabata S, Davie EW. A microsomal endopeptidase from liver with substrate specificity for processing proproteins such as the vitamin K-dependent proteins of plasma. *J Biol Chem* 1992; 267: 10331–10336.
14. Wise R, Barr PH, Wong PA, *et al.* Expression of a human proprotein processing enzyme: current cleavage of the von Willebrand factor precursor at a paired basic amino acid site. *Proc Natl Acad Sci USA* 1990; 87: 9378–9382.
15. Wasley L-C, Rehemtulla A, Bristol JA, *et al.* PACE/Furin can process the vitamin K-dependent pro-factor IX precursor within the secretory pathway. *J Biol Chem* 1993; 268: 8458–8465.
16. Meulien P, Balland A, Lepage P, *et al.* Increased biological activity of a recombinant factor IX variant carrying alanine at position +1. *Protein Eng* 1990; 3: 629–633.
17. Cheung A, Suttie JW, Bernatowicz M. Vitamin K-dependent carboxylase: structural requirements for propeptide activation. *Biochim Biophys Acta* 1990; 1039: 90.
18. Furie B, Furie BC. Molecular basis of γ-carboxylation: role of the propeptide in the vitamin K-dependent proteins. *Ann NY Acad Sci* 1991; 614: 1–10.
19. Foster DC, Rudinski MS, Schach BG, *et al.* Propeptide of human protein C is necessary for γ-carboxylation. *Biochemistry* 1987; 26: 7003–7011.
20. Diuguid DL, Rabiet MJ, Furie BC, *et al.* Molecular basis of haemophilia B: a defective enzyme due to an unprocessed propeptide is caused by a point mutation in the factor IX precursor. *Proc Natl Acad Sci USA* 1986; 83: 5803–5807.
21. Bentley AK, Rees DJG, Rizza C, *et al.* Defective propeptide processing of blood clotting factor IX caused by mutation of arginine to glutamine. *Cell* 1986; 45: 343–348.
22. Wu SM, Cheung WF, Frazier D, *et al.* Cloning and expression of the cDNA for human γ-glutamyl carboxylase. *Science* 1991; 254: 1634–1636.
23. Rehemtulla A, Roth DA, Wasley L, *et al.* In vitro and in vivo functional characterization of bovine vitamin K-dependent γ-carboxylase expressed in Chinese hamster ovary cells. *Proc Natl Acad Sci USA* 1993; 90: 4611–4615.
24. Kaufman RJ, Wasley LC, Furie, BC, *et al.* Expression, purification, and characterization of recombinant factor IX synthesized in Chinese hamster ovary cells. *J Biol Chem* 1986; 261: 9622–9628.
25. Kurachi K. Recombinant antihaemophilic factors. In: Goldstein J, ed. *Biotechnology of Blood*, Boston: Butterworth-Heinemann, 1991: 177–195.
26. Balland A, Faure T, Carvallo D, *et al.* Characterization of two differently processed forms of human recombinant factor IX synthesized in CHO cells transformed with a polycistronic vector. *Eur J Biochem* 1988; 172: 565–572.
27. Verweij CL, Hart M, Pannekoek H. Expression of variant von Willebrand factor (vWF) cDNA in heterologous cells: requirement of the pro-polypeptide in vWF multimer formation. *EMBO J* 1987; 6: 2885–2890.
28. Wise RJ, Pittman DD, Handin RI, Kaufman RJ, Orkin SH. The propeptide of the von Willebrand factor independently mediates the assembly of von Willebrand multimers. *Cell* 1988; 52: 229–236.
29. Sadler JE. von Willebrand factor. *J Biol Chem* 1991; 266: 22777–22780.
30. Giannelli F, Green PM, High KA, *et al.* Haemophilia B: database of point mutations and short additions and deletions. *Nucleic Acids Res* 1992; 20: 2027–2063.
31. Thompson AR. Molecular biology of the haemophilias. *Prog Haemostasis Thromb* 1991; 10: 175–214.
32. Wang NS, Zhang M, Thompson AR, *et al.* Factor $IX_{Chicago}$: a new mutation in the calcium binding domain of factor IX resulting in severe haemophilia B. *Thromb Haemostas* 1990; 63: 24–26.
33. Stenflo J. Structure-function relationships of epidermal growth factor modules in vitamin K-dependent clotting factors. *Blood* 1991; 78: 1637–1651.
34. Astermark J, Bjork I, Ohlin A-K, *et al.* Structural requirements for Ca^{2+} binding to the γ-carboxyglutamic acid and epidermal growth factor-like regions of factor IX. *J Biol Chem* 1991; 266: 2430–2437.
35. Tulinsky A. The structure of domains of blood proteins. *Thromb Haemostas* 1991; 66: 16–30.
36. Soriano-García M, Padmanabhan K, de Vos AM, *et al.* The Ca^{2+} ion and membrane binding structure of the Gla domain of Ca-prothrombin fragment 1. *Biochemistry* 1992; 31: 2554–2566.

37. Wolberg AS, Cheung W-F, Stafford DW. Characterization of the functional roles of γ-carboxylglutamic acid (Gla) residues 20 and 21 in human factor IX. *Thromb Haemostas* 1993; 69: 614.
38. Cheung W-F, Hamaguchi N, Smith KJ, *et al.* The binding of human factor IX to endothelial cells is mediated by residues 3–11. *J Biol Chem* 1992; 267: 20529–20533.
39. Huang LH, Ke X-H, Sweeney W, *et al.* Calcium binding and putative activity of the epidermal growth factor domain of blood coagulation factor IX. *Biochem Biophys Res Commun* 1989; 160: 133–139.
40. Selander M, Persson E, Stenflo J, *et al.* ^{1}H NMR assignment and secondary structure of the Ca^{2+}-free form of the amino-terminal epidermal growth factor-like domain in coagulation factor X. *Biochemistry* 1990; 29: 8111–8118.
41. Handford PA, Baron M, Mayhew M, *et al.* The first EGF-like domain from human factor IX contains a high-affinity calcium binding site. *EMBO J* 1990; 9: 475–480.
42. Fernlund P, Stenflo J. β-hydroxyaspartic acid in vitamin K-dependent proteins. *J Biol Chem* 1983 258: 12509–12512.
43. Hase S, Nishimuru H, Kawabata S-I, *et al.* The structure of (Xylose)$_2$Glucose-O-Serine 53 found in the first epidermal growth factor-like domain of bovine blood clotting factor IX. *J Biol Chem* 1990; 265: 1858–1861.
44. Derian CK, VanDusen W, Przysiecki CT, *et al.* Inhibitors of 2-ketoglutarate dependent dioxygenases block aspartyl β-hydroxylation of recombinant human factor IX in several mammalian expression systems. *J Biol Chem* 1989; 264: 6615–6618.
45. Johnson AE, Esmon NL, Lane TM, *et al.* Structural changes required for activation of protein C are induced by Ca^{2+} binding to a high affinity site that does not contain γ-carboxyglutamic acid. *J Biol Chem* 1983; 258: 5554.
46. Davis LM, McGraw RA, Ware JL, *et al.* Factor IX$_{\text{Alabama}}$: a point mutation in a clotting protein results in haemophilia B. *Blood* 1987; 69: 140–143.
47. Lozier JN, Monroe DM, Stanfield-Oakley S, *et al.* Factor IX$_{\text{New London}}$: Substitution of Proline for glutamine at position 50 causes severe haemophilia B. *Blood* 1990; 75: 1097–1104.
48. Spitzer SG, Kuppuswamy MN, Saini R, *et al.* Factor IX$_{\text{Hollywood}}$: Substitution of Pro55 by Ala in the first epidermal growth factor-like domain. *Blood* 1990; 76: 1530–1537.
49. Lin SW, Smith KJ, Welsch D, *et al.* Expression and characterization of human factor IX and factor IX-factor X chimeras in mouse C127 cells. *J Biol Chem* 1990; 265: 144–150.
50. Miyata T, Nishimura H, Suehiro K, *et al.* The 2nd EGF-like domain of factor IX is required for interaction with factor VIII. *Thromb Haemostas* 1991; 65: 817.
51. Hertzberg MS, Ben-Tal O, Furie B, *et al.* Construction, expression, and characterization of a chimera of factor IX and factor X: the role of the second epidermal growth factor domain and serine protease domain in factor Va binding. *J Biol Chem* 1992; 267: 14759–14766.
52. Noyes CM, Griffith MJ, Roberts HR, *et al.* Identification of the molecular defect in factor IX$_{\text{Chapel Hill}}$: substitution of histidine for arginine at position 145. *Proc Natl Acad Sci USA* 1983; 80: 4200–4202.
53. Liddell MB, Peake IR, Taylor SAM, *et al.* Factor IX$_{\text{Cardiff}}$: a variant factor IX protein that shows abnormal activation is caused by an arginine to cysteine substitution at position 145. *Br J Haematol* 1989; 72: 556–560.
54. Di Scipio RG, Kurachi K, Davie EW. Activation of human factor IX (Christmas factor). *J Clin Invest* 1978; 61: 1528–1538.
55. Bajaj SP, Spitzer SG, Welsh WJ, *et al.* Experimental and theoretical evidence supporting the role of Gly363 in blood coagulation factor IXa (Gly193 in chymotrypsin) for proper activation of the proenzyme. *J Biol Chem* 1990; 265: 2956–2961.
56. Usharani P, Warn-Cramer BJ, Kasper CK, *et al.* Characterization of three abnormal factor IX variants (Bm Lake Elsinore, Long Beach, and Los Angeles) of Haemophilia-B. *J Clin Invest* 1985; 75: 76–83.
57. Bottema CDK, Ketterling RP, Li S, *et al.* Missense mutations and evolutionary conservation of amino acids: evidence that many of the amino acids in factor IX function as "spacer" elements. *Am J Hum Genet* 1991; 49: 820–838.
58. Bajaj SP, Sabarwal AK, Gorka J, *et al.* Antibody-probed conformational transitions in the protease domain of factor IX upon calcium binding and zymogen activation: putative high affinity calcium binding site in the protease domain. *Proc Natl Acad Sci USA* 1992; 89: 152–156.
59. Sinha U, Wolf DL. Carbohydrate residues modulate the activation of coagulation factor X. *J Biol Chem* 1993; 268: 3048–3051.
60. Choo KH, Gould KG, Rees DJG, *et al.* Molecular cloning of the gene for human anti-haemophilic factor IX. *Nature* 1982; 299: 178–180.
61. Chance PF, Dyer KA, Kurachi K, *et al.* Regional localization of human factor IX gene by molecular hybridization. *Hum Genet* 1983; 65: 207–208.
62. Purrello M, Alhadeff B, Esposito D, *et al.* The human genes for haemophilia A and haemophilia B flank the X chromosome fragile site at Xq27.3. *EMBO J* 1985; 4: 725–729.
63. Camerino G, Grzeschik KH, Jaye M, *et al.* Regional localization on the human X chromosome and polymorphism of the coagulation factor IX gene (haemophilia B locus). *Proc Natl Acad Sci USA* 1984; 81: 498–502.
64. Bottema CD, Bottema MJ, Ketterling RP, *et al.* Why does the human factor IX gene have a G + C content of 40%? *Am J Hum Genet* 1991; 49: 839–850.
65. Foster DJ, Yoshitake S, Davie EW. The molecular sequence of the gene for human protein C. *Proc Natl Acad Sci USA* 1985; 82: 4673–4677.
66. Schmidel DK, Tatro AV, Phelps LG, *et al.* Organization of the human protein S genes. *Biochemistry* 1990; 29: 7845–7852.
67. Degen SJF, Davie EW. Nucleotide sequence of the gene for human prothrombin. *Biochemistry* 1987; 26: 6165–6177.
68. Hojrup P, Jensen MS, Petersen TE. Amino acid sequence of bovine protein Z: a vitamin K-dependent serine protease homology. *FEBS Lett* 1985; 184: 333–338.
69. Seijima H, Hayashi T, Deyashiki Y, *et al.* Primary structure of vitamin K-dependent human protein Z. *Biochem Biophys Res Commun* 1990; 171: 661–666.

70. Leytus SP, Foster DC, Kurachi K, *et al.* Gene for human factor X: a blood coagulation factor whose gene organization is essentially identical with that of factor IX and protein C. *Biochemistry* 1986; 25: 5098–5102.
71. O'Hara PJ, Grant FJ, Haldeman BA, *et al.* Nucleotide sequence of the gene coding for human factor VII, a vitamin K-dependent protein participating in blood coagulation. *Proc Natl Acad Sci USA* 1987; 84: 5158–5162.
72. Hay CW, Robertson KA, Young SN, *et al.* Use of Bam HI polymorphism in the factor IX gene for the determination of haemophilia B carrier status. *Blood* 1986; 67: 1508.
73. Winship PR, Anson DS, Rizza CR, *et al.* Carrier detection in haemophilia B using two further intragenic restriction fragment length polymorphisms. *Nucleic Acids Res* 1984; 12: 8861–8872.
74. Camerino G, Grzeschik KH, Jaye M, *et al.* Regional localization on the human X-chromosome and polymorphism of the coagulation factor IX gene (haemophilia B locus). *Proc Natl Acad Sci USA* 1984; 81: 498–502.
75. McGraw RA, Davis LM, Noyes CM, *et al.* Evidence for a prevalent dimorphism in the activation peptide of human coagulation factor IX. *Proc. Natl Acad Sci USA* 1985; 82: 2847–2851.
76. Tanimoto M, Hamaguchi M, Matsushita T, *et al.* A new marker (F9–192) of the factor IX gene detected by denaturing gel electrophoresis. *Blood* 1989; 74: 251a.
77. Driscoll MC, Dispenzieri A, Tobias E, *et al.* A second Bam HI DNA polymorphism and haplotype association in the factor IX gene. *Blood* 1988; 72: 61–65.
78. Camerino G, Oberle I, Drayana D, *et al.* A new Msp I restriction fragment length polymorphism in the haemophilia B locus. *Hum Genet* 1985; 71: 79–81.
79. Winship PR, Rees DJG, Alkan M. Detection of polymorphisms at cytosine phosphoguanadine dinucleotides and diagnosis of haemophilia B carriers. *Lancet* 1989; 25: 631–634.
80. Reiner AP, Thompson AR. An Hha I polymorphism is present in factor IX genes of Asian subjects. *Hum Genet* 1990; 86: 87–88.
81. Mulligan L, Holden JJA, White BN. A DNA marker closely linked to the factor IX (haemophilia B) gene. *Hum Genet* 1987; 75: 381–383.
82. Hofker MH, Bergen AAR, Skraastad MI, *et al.* Efficient isolation of X chromosome-specific single-copy probes from a cosmid library of a human X/hamster hybrid-cell line: mapping of new probes close to the locus for X-linked mental retardation. *Am J Hum Genet* 1987; 40: 312–328.
83. Kurachi K, Chen S-H. Molecular genetic analyses of congenital disorders of coagulation factors. In: Bern MM, Frigoletto F, eds. *Hematologic Disorders in Maternal–Fetal Medicine*. New York: Wiley-Liss, Inc., 1989: 449–466.
84. Sarkar G, Paynton C, Sommer SS. Segments containing alternating purine and pyrimidine dinucleotides: patterns of polymorphism in humans and prevalence throughout phylogeny. *Nucleic Acids Res* 1991; 19: 631–636.
85. Kaplan DJ, Duncan CH. Novel short interspersed repeat in human DNA. *Nucleic Acids Res* 1990; 18: 192.
86. Chen S-H, Scott CR. Recombination between two 14-bp homologous sequences as the mechanism for gene deletion in factor $IX_{Santo\ J}$. *Am J Hum Genet* 1990; 47: 1020–1022.
87. Vidaud M, Vidaud D, Siguret V, *et al.* Mutational insertion of an Alu sequence causes haemophilia B. *Am J Hum Genet* 1989; 45: A226.
88. Chen S-H, Scott CR, Edson R, *et al.* An insertion within the factor IX gene for haemophilia$_{El\ Salvador}$. *Am J Hum Genet* 1988; 42: 581–584.
89. Kazazian HH, Wong C, Youssoufian H, *et al.* Haemophilia A resulting from *de novo* insertion of L1 sequences represents a novel mechanism for mutation in man. *Nature* 1988; 332: 164–166.
90. Youssoufian H, Kazazian HH, Patel A, *et al.* Mild haemophilia A associated with a cryptic donor splice site mutation in intron 4 of the factor VIII gene. *Genomics* 1988; 2: 32–36.
91. Toole JJ, Pittman DD, Orr EC, *et al.* A large region (X95 kDa) of human factor VIII is dispensable for *in vitro* procoagulant activity. *Proc Natl Acad Sci USA* 1986; 83: 5939–5942.
92. Pittman DD, Alderman EM, Tomkinson KN, *et al.* Biochemical, immunological, and *in vivo* functional characterization of B-domain-deleted factor VIII. *Blood* 1993; 81: 2925–2935.
93. Koeberl DD, Bottema CD, Buerstedde J-M, *et al.* Functionally important regions of the factor IX gene have a low rate of polymorphism and a high rate of mutations in the dinucleotide CpG. *Am J Hum Genet* 1989; 45: 448–457.
94. Sommer SS. Assessing the underlying pattern of human germline mutations: lesions from the factor IX gene. *FASEB J* 1992; 6: 2767–2774.
95. Higuchi M, Kazazian HH, Jr, Kasch L, *et al.* Molecular characterization of severe haemophilia A suggests that about half the mutations are not within the coding regions and splice junctions of the factor VIII gene. *Proc Natl Acad Sci USA* 1991; 88: 7405–7409.
96. Ketterling RP, Bottema CD, Koeberl DD, *et al.* T296IM, a common mutation causing mild haemophilia B in the Amish and others: founder effect, variability in factor IX activity assays and rapid carrier detection. *Hum Genet* 1991; 87: 333–337.
97. Taylor SAM, Deugan KV, Lillicrap DP. Somatic mosaicism and female-to-female transmission in a kindred with haemophilia B. *Proc Natl Acad Sci USA* 1991; 88: 39–42.
98. Nisen P, Stamberg J, Ehrenpreis R, *et al.* The molecular basis of severe haemophilia B in a girl. *N Engl J Med* 1986; 315: 1139–1142.
99. Salier J-P, Hirosawa S, Kurachi K. Functional characterization of the 5'-regulatory region of human factor IX gene. *J Biol Chem* 1990; 265: 7062–7068.
100. Sarkar G, Sommer SS. Access to a messenger RNA sequence or its protein product is not limited by tissue or species specificity. *Science* 1989; 244: 331–334.
101. Hassan HJ, Leonardi A, Chelucci C, *et al.* Blood coagulation factors in human embryonic-fetal development: preferential expression of the FVII/tissue factor pathway. *Blood* 1990; 76: 1158–1164.
102. Kisker CT, Robillard JE, Clarke WR. Development of blood coagulation—a fetal lamb model. *Pediatr Res* 1981; 15: 1045–1050.

141. Axelrod JH, Read MS, Brinkhous KM, Verma IM. Phenotypic correction of factor IX deficiency in skin fibroblasts of haemophilic dogs. *Proc Natl Acad Sci USA* 1990; 87: 5173–5177.
142. Yao S-N, Wilson JM, Nabel EG, *et al.* Expression of human factor IX in rat capillary endothelial cells: Toward somatic gene therapy for haemophilia B. *Proc Natl Acad Sci USA* 1991; 88: 8101–8105.
143. Armentano D, Thompson AR, Darlington G, *et al.* Expression of human factor IX in rabbit hepatocytes by retrovirus-mediated gene transfer: potential for gene therapy of haemophilia B. *Proc Natl Acad Sci USA* 1990; 87: 6141–6145.
144. Yao S-N, Kurachi K. Expression of human factor IX in mice after injection of genetically modified myoblasts. *Proc Natl Acad Sci USA* 1992; 89: 3357–3361.
145. Gerrard AJ, Hudson DL, Brownlee GG, *et al.* Towards gene therapy for haemophilia B using primary human keratinocytes. *Nature Genet* 1993; 3: 180–183.
146. Yao S-N, Kurachi K. A simple treatment of serum for precise determination of recombinant factor IX in the culture media. *BioTechniques* 1991; 12: 525–526.
147. Scharfmann R, Axelrod JH, Verma IM. Long-term *in vivo* expresssion of retrovirus-mediated gene transfer in mouse fibroblast implants. *Proc Natl Acad Sci USA* 1991; 88: 4626–4630.
148. Hseuh JL, Lu DR, Zhou JM, *et al.* Gene therapy for haemophilia B using fibroblasts. Part I. *Sci China* (Ser B), 1993; 23: 53–60.
149. Yao S-N, Wilson JM, Nabel EG, *et al.* Expression of human factor IX in rat capillary endothelial cells: Toward somatic gene therapy for haemophilia B. *Proc Natl Acad Sci USA* 1991; 88: 8101–8105.
150. Ponder KP, Gupta S, Feland F, *et al.* Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. *Proc Natl Acad Sci USA* 1991; 88: 1217–1221.
151. Kay, MA, Baley P, Rothenberg S, *et al.* Expression of human α_1-antitrypsin in dogs after autologous transplantation of retroviral transduced hepatocytes. *Proc Natl Acad Sci USA* 1992; 89: 89–93.
152. Perkol T, Lindberg GL, Chen J, *et al.* Regulation of the phosphenolpyruvate carboxykinase/human factor IX gene introduced into the livers of adult rats by receptor-mediated gene transfer. *FASEB J* 1993; 7: 1081–1091.
153. Kay MA, Rothenberg S, Lande CN, *et al. In vivo* gene therapy of haemophilia B: sustained partial correction in factor IX-deficient dogs. *Science* 1993; 263: 117–119.

APPENDIX C: RELATED PROCEEDINGS

Not applicable